REMARKS

Status of the Claims

Claims 1-38 were pending. Claims 17 and 19-23 have been canceled without prejudice or disclaimer. Claims 1-5, 7-12, 14-16, 18, 24-32 and 35-36 have been amended. New claims 39 and 40 have been added. No new matter has been added. Claims 1-16, 18 and 24-39 are under examination.

Claims Amendments

Claims 1 and 35 have been amended to recite "wherein the athletic performance is selected from at least one of sprint performance, endurance performance, power performance and strength performance." Support for these amendments can be found throughout the specification but at least at paragraphs [0021], [0027]-[0031], [0040], [0041], [0096], [0126] and [0127] and Examples 1-3 of the Application as originally filed. These amendments were made without prejudice or disclaimer in effort to further prosecution.

Claim 1 has been amended to delete "the" in order to correct antecedent basis of ACTN3.

Claims 1-5, 7-12, 14-16, 18, 24-32 and 35-36 have been amended to recite "mammal" instead of "individual" to clarify the claims. Support for these amendments can be found throughout the specification but at least at paragraphs [0032] to [0040] and Examples 1-3 of the Application as originally filed. These amendments were made without prejudice or disclaimer in effort to further prosecution.

New claims 39 and 40 have been added. Support for this claim can be found throughout the specification but at least at paragraphs [0012], [0032], [0040], [0042], [0044], [0115] and [0116] and Claims 3, 25, 26 and 30 of the Application as originally filed.

Drawings

Page 3 paragraph 2 of the Action recites "[t]he disclosure is objected to because it contains an embedded hyperlink...Table 3 of the figures contains a hyperlink..." The Applicant respectfully submits that Table 3 of the Application as filed has been amended to delete the hyperlink and a clean copy of Table 3 is provided herein. The Applicant respectfully requests

removal of the objection.

Amendments to the Specification

Page 3 paragraph 3 of the Action recites "[t]he disclosure is objected to because...page 7, paragraph 0027...refers to Table 3 for ethnic differences." The Applicant has corrected the reference to "Table 3" to now recite "Table 6." The Applicants respectfully refer to page 2 of this response entitled "Amendments to the Specification." The Applicant request removal of the objection to the specification.

Page 3 paragraph 4 of the Action recites "Page 14, line 2 contains a hyperlink." The Applicant submits that the specification has been amended to remove the hyperlink in paragraph [0045] of the Application as originally filed, please see page 2 of this response. The Applicant requests removal of the objection.

Rejection of Claims Under 35 USC § 112, First Paragraph, Enablement

Claims 1-18 and 24-32 were rejected on page 4 of the Action under 35 USC § 112, 1st paragraph for lack of enablement. On page 4, the Action asserts, that "because the specification, while being enabling for determining sprint performance in males by the presence of ACTN3 577RR genotype, does not reasonably provide enablement for predicting athletic performance in males or females..." The Applicant respectfully disagrees with this rejection.

On pages 5-7, the Action asserts certain teachings by the specification. As recited above, the Action asserts that "while being enabling for determining sprint performance in males...[the specification] does not reasonably provide enablement for predicting athletic performance in males or females.." On page 7, the Action asserts that "there is no significant difference between elite athletes and controls, although there *was* a difference in sprint athletes both male and *female*...Further the specification teaches there was a *significant* allele frequency difference between elite endurance athletes and elite sprint athletes."

The Applicant respectfully submits that the experimental data disclosed in the instant application supports the athletic performances disclosed in the instant claims as amended. Independent claims 1 and 35 have been amended to recite "wherein the athletic performance is selected from at least one of sprint performance, endurance performance, power performance and

strength performance." The data disclosed in the instant application support that ACTN3 genotypes can be used to predict athletic performance in an individual selected from at least one of sprint performance, endurance performance, power performance and strength performance, elements of amended independent claims 1 and 35.

The Applicants respectfully submit that support for analyzing ACTN3 genotypes to predict sprint performance, endurance performance, power performance or strength performance in an individual other than a human (e.g. horse, dog or camel) is disclosed at least in paragraphs [0032] to [0040] of the application as originally filed.

On page 7, the Action asserts that Mills et al (Molecular Genetics (2001) Volume 10 pages 1335-1346; hereinafter "Mills" "thus teaches that neither the 577X allele *nor* the ACTN3 gene is predictably found in "any" subject." The Applicant respectfully disagrees with this assertion. The Applicant respectfully points to page 1340 where Mills recites "we have shown that α-Actinin-3 *may* not be functionally redundant in mouse *skeletal* muscle. α-Actinin-3 is the predominant isoform in postnatal mouse muscle..." and pages 1341-42 where Mills recites "[d]espite the apparent lack of two genes in birds, the mammalian ACTN2 and ACTN3 genes appear to have arisen from a gene duplication that occurred substantially earlier than the divergence of birds and mammals...In mammals both copies of the gene have survived, and our comparison shows that the genes have been highly conserved throughout mammalian evolution...The complete overlap of ACTN3 function by ACTN2, apparent in humans, may have arisen relatively recently, although the similarity of expression patterns between humans and baboons suggest that it is at least as old as the time of separation of these species..."

On page 8, the Action asserts that Gene Card teaches "ACTN3 homologs have only been found in dog, fruit fly, zebrafish, mouse, chimpanzee and rat....while listing 32 species which a homolog of ACTN3 has not been found." The Applicant respectfully submits that of the "32 species" which the Action asserts "a homolog of ACTN3 has not been found," include pig, cow, chicken, worm, 4 species of yeast, fungus, 2 kinds of frogs, trout, soybean ,cress, barley, tomato, sugarcane, pine, rice, corn, wheat, grape, bread mold, sea squirt, amoeba, trunc, malaria parasite, schistosome parasite, sorghum, and toxoplasmosis. The Applicant respectfully submit that of the 32 organisms that were asserted in the Action, only *2 mammals*, cow and pig, were "no similarity-to-human data found for ACTN3" but 4 mammals were found to have the ACTN3,

dog, mouse, chimpanzee and rat. The other **29** of 32 organisms listed in the "no similarity-to-human data found for ACTN3" were *amphibians, fish*, lower organisms such as *parasites* and even *single-celled* organisms.

On page 8, the Action asserts that "elite distance runners from Ethiopia and Kenya did not significantly differ from those of their respective control population..." see (Yang, et al (Med. Sci. Sport and Exercise (2005) vol. 37, s42; hereinafter "Yang"). The Action also asserts that Yang "teaches, "this polymorphism does not contribute significantly to the phenomenal success of elite East African endurance runners." The Applicant respectfully reject this assertion.

The Applicants respectfully submit that the sample size of Yang fails to support the assertion of the Action. The Applicant respectfully submit in Yang only about 10% of the Ethiopian's tested (about 13 individuals) had the XX genotype, whereas only 1% of the Kenyans tested (about 3 individuals) had the XX genotype. Thus, the Applicant submits that the sample size having the XX genotype is not significant and thus no conclusion can be made from Yang.

On page 8, the Action asserts that Moran et al (European Journal of Human Genetics (2007) volume 15, pages 88-93; hereinafter "Moran") "teaches in a study of 992 *adolescent* [subjects between the ages of 11-18] Greeks the presence of the 577R allele resulted in a significant association with sprint times over 40m in males, but not females...We found no evidence that the R577X genotype is associated with endurance or obesity related genotypes...that the 577X allele is not predictably associated with endurance performance." The Applicant respectfully disagrees.

The Applicant respectfully submit that the assertion in the action is unreasonable in light of the nature of the samples analyzed by Moran. In addition, the Applicant submits that the data in Moran does not show results following suitable training regimes to be able to draw a conclusion that a particular genotype is associated with athletic performance or capable of detecting potential performance following suitable training. In fact, page 92 under "Discussion," Moran recites..."but the data do not reveal an association in a large sample of unselected adolescent female subjects. This is probably explained by differenced between untrained adolescents and elite sprint athletes in terms of their exercise environments and muscle physiology, which may modify the interaction with genotype at the R577X polymorphism." In addition, page 93 of Moran recites "In this nonelite population there was no evidence, however,

for the association with other power/strength-related phenotypes involving single muscle contraction events..." The Applicants submit that Moran studied 40-meter sprint performance in adolescent females and that a more appropriate test would involve looking at the ACTN3 genotype in the females and exposing the individuals to suitable training regimes to maximize their sprint potential. The Applicant points out that the 40-meter sprint performance in females of Moran have a greater likelihood of excelling at sprint performance but will not realize the potential without appropriate training.

The Applicant asserts that the instant application discloses that a strong association was identified between an ACTN3 genotype and sprint performance in females. In addition, the Applicants submit that *elite* athletes and suitable controls were used for studies disclosed in the instant Application, not non-elite adolescents.

On page 9, the Action asserts that Lucia (International Journal of Sports Medicine (2006) volume 27, pages 880-884; hereinafter "Lucia") "studied the frequency of the ACTN3 genotype in a group of 50 top level professional cyclists and 52 Olympic class endurance runners...[and]...there was no significant differences between elite runners and cyclists and the R577X genotype...[and]..it would be unpredictable to associate the R577X genotype with improved endurance performance." The Applicant submits that on page 883, left column Lucia recites "Our groups had small sample sizes, which *decreases* the statistical power of comparisons...Another potential limitation comes from the fact that we did not corroborate our findings in a group of female athletes...Pioneering research...has indeed reported significantly higher frequency distribution of the XX genotype in female endurance athletes than in controls...Further the very high speeds and near maximal intensities at which endurance events are currently performed by top-level runners probably requires the ability to recruit type II fibres, i.e. expressing α-actinin-3. For instance, contemporary running velocities in 200 and 10000m events are such that more and more top-level distance runners are able to run 1500 m in less than 3 min 35 s." The Applicant notes that only small numbers of samples were analyzed and that this samples size fails to support the claimed assertion by the Action, the reference itself points to this shortcoming.

The Applicant respectfully submits that other submissions of reliable scientific evidence support the instant Application. For example, Niemi and Majamaa (Eur. J. Hum. Genet. (2005)

13:965-969; attached in Appendix A) analyzed ACTN3 genotypes in Finnish elite endurance and sprint athletes. Figure 1 illustrates that the frequency of the 577XX was higher in endurance athletes than the controls or sprint athletes, whereas the frequency of the RR genotype is higher in sprint athletes when compared to control and endurance athletes. In addition, Papadimitriou et al (Int J Sports Med (2007); attached in Appendix A) analyzed elite (not non-elite adolescents) Greek track and field athletes and observed a greater frequency of the 577RR genotype in power oriented athletes than the general population. In Roth et al (Med(2007) S280; attached in Appendix A) shows that the 577XX genotype is negatively correlated with elite strength athletes. Of particular relevance is MacArthur et al (Nature Genetics (2000) 39:1261-1265; hereinafter MacArthur; attached in Appendix A) that illustrates that the 577XX genotype provides an advantage for endurance activity by shifting the metabolic pathway of fast twitch muscle fibers to a slower but more efficient aerobic pathway. Thus, the XX genotype in the transgenic mice produced by MacArthur clearly associated with an increased endurance performance. Thus, the Applicant submits that significant scientific data in the specification and other post-filing references clearly support an association between the ACTN3 genotype and sprint, power, strength and endurance performance, elements of independent claims 1 and 35.

The Applicant respectfully submit that the claims have been amend to recite "mammal" versus "individual" in the interest of advancing prosecution and to overcome the objection on page 10 of the Action to "any" individual. It has been well established that the ACTN3 orthologs are found in most, if not all mammals, and other animal species (see also paragraph [0038] of the instant application as filed). Certain particular embodiments of the instant application are directed toward humans, horse, dogs and camels. The Applicant submit that undue experimentation would not be required in order to assess athletic performance in a mammal by analyzing one or more genetic variations in the α–actinin-3 (ACTN3) gene of a selected mammal as a factor in predicting athletic performance of the mammal related to sprint, power, strength and endurance performance. The Applicant respectfully requests removal of the rejections.

Rejection of Claims Under 35 USC § 112, First Paragraph, Written Description

Claims 1-16, 18, and 24-38 were rejected on page 12 of the Action under 35 USC § 112, 1st paragraph for "failing to comply with the written description requirement." On page 12, the

Action asserts, that "[t]he claim(s) contain subject matter, which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." The Applicant respectfully disagrees.

The Applicant submits that scope of the claims as they relate to the nature of the ACTN3 gene, and the polymorphisms thereof, fulfills the written description requirements as detailed previously in the response. The Applicant was in possession of the claimed invention at the time of filing. The instant application discloses that a sample from a mammal is analyzed for "the presence of one or more genetic variations in **α–actinin-3 (ACTN3) gene**" found in the 11q13.1 not the whole chromosome. In certain embodiments, samples from a mammal are selected to predict "athletic performance based on the presence of the one or more genetic variations wherein the athletic performance is selected from at least one of sprint performance, endurance performance, power performance and strength performance." In other embodiments, a genetic variation may include 1747 C>T single nucleotide polymorphism (SNP) in the ACTN3 gene. In addition, other embodiments concern assessing 577XX genotype of the mammal (e.g. human, dog, horse and camel). Therefore, the Applicant submits that the specification supports the claims under examination. The Applicant respectfully requests removal of the rejection.

Rejection of Claims Under 35 USC 103(a)

On page 15, the Action recites that "[c]laims 1-2, 4-14, 15-16, 24-37 are rejected under 35 U.S.C. 103(a) as being unpatentable over North (Nature Genetics (199) volume 21 pages 353-354; hereinafter "North") in view of Costill et al (Journal of Applied Physiology (1976) volume 40, pages 149-153; hereinafter "Costill").

The Action asserts that North "teaches the a-actinin-3…is expressed only in type 2b and 50% type 2a fibers…that ACTN3 577XX may be associated with the preferential loss of type 2 fibers…taking biopsies from individuals ..genotyping at the ACTN3 locus…..screening…typing of a sample." The Action further asserts that Costill "teaches that training dictates ultimate capacity for endurance, success in sprint or distance running is predetermined by fiber type compositions…Costill inherently teaches that sprinters thus had 72.6% fast twitch (type 2) fibers

while distance runners had 30.6% fast twitch (type 2)...that increased fast twitch...is associated with increased sprint performance..."

The Office Action has taken the position that one skilled in the art at the time the invention was made would be motivated to combine the cited references. Applicants respectfully traverse this rejection. The cited art does not support this position. Rejection of claims under 35 USC§103 is improper unless each and every element of the claimed invention is disclosed in one of the cited prior art references. [*In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991)].

The Action asserts that it would have been "prima facie obvious to one of ordinary skill in the art at the time the invention was made to combine the genotyping assay of North to screen individuals for the presence of the 577RR or 577RX genotype indicative of type 2 fibers and predict athletic performance with the teaching of Costill that type 2 fibers are related to improved performance in sprinting events."

The Applicant submits that the Action incorrectly interprets North. More specifically, the Action asserts that North teaches that "ACTN3 577XX may be associated with a preferential loss of type 2 fibers" and as support refers to Applicant to the third column, last paragraph of page 353. This assertion is inaccurate. North recites that "[h]omozygosity for the stop codon at position 577 was identified in 46 of 48 (96%) in which α-actinin-3 staining was negative." This meant that the remaining two patients possessed genes encoding the full length protein but that the protein could not be detected. The authors then went on to explain that these two remaining individuals were negative using the relatively insensitive protein assay because the individuals had less than 5% type 2 fibers.

The Applicant asserts that North does not teach or suggest that individuals with a 577XX genotype have a preferential loss of type 2 fibers. In fact, North discloses that the genotype of ACTN3 did not alter fiber-type distribution in control samples (see North page 353 last sentence continued onto page 354). Thus, contrary to assertions in the Action, there is absolutely no evidence that North teaches or suggests that the presence of the 577RR or 577RX genotype is indicative of type 2 fibers. Furthermore, post-filing data has shown that the 577RR genotype is associated with the location and number of a sub-population of type 2 fibers (see Vincent et al

(2007); attached in Appendix A) which clearly demonstrates that the assertion in the Action, that 577XX genotype have a preferential loss of type 2 fibers, is incorrect.

North fails to disclose all the elements of the instant claims. For example, North fails to disclose analyzing the ACTN3 gene to predict athleticism, formulate training programs or select a sprint or endurance sport for an individual, elements of amended independent claim1 and 35. North demonstrates the existence of mutations amongst the general population but does not disclose the use of identifying genetic variations and assessing athletic ability or maximizing ability in an individual based on the presence or absence of *any* of the disclosed genetic variations in the ACTN3 gene. The Applicant submits that the rejections under 103 raised in the Action are based on a misinterpretation of North. North does not teach the loss, preferential or otherwise, in type 2 fibers of any specific genotype of the ACTN3 gene.

The rejections by the Action are based on North in combination with other references. Therefore, once the impropriety of using North in the rejections is established, all of the rejections based on North in combination with other references must fall.

A prima facie case of obviousness requires: 1) some suggestion or motivation, either in the references themselves of in the knowledge generally available in the art, to modify the reference or combine the teachings; 2) a reasonable expectation of success; 3) the prior art reference or references must teach or suggest all the claim limitations. [MPEP 2142]

As discussed above, North does not provide sufficient suggestion or motivation to combine the references because North does not teach or suggest all the claim limitations, notably that ACTN3 genotypes can be used for "predicting athletic performance based on the presence of the one or more genetic variations wherein the athletic performance is selected from at least one of sprint performance, endurance performance, power performance and strength performance." This deficiency is not corrected by any of the other cited references alone or in combination.

There is no disclosure in any of the other cited references of "predicting athletic performance based on the presence of the one or more genetic variations wherein the athletic performance is selected from at least one of sprint performance, endurance performance, power performance and strength performance." The Action is unable to point to any disclosure in Costill that discloses these elements of the claimed subject matter. Rejection under §103 is

improper. The Applicants submit that amended independent claims 1 and 35 are in condition for allowance.

CONCLUSION

For the reasons stated above, Applicant asserts that claims 1-16, 18, 24-39 are in condition for allowance.

Respectfully submitted,

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ARTICLE

Mitochondrial DNA and ACTN3 genotypes in Finnish elite endurance and sprint athletes

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Differences in ACTN3 (alpha-actinin 3) genotypes have been reported among endurance and power athletes. Elite athletic performance in endurance sports should also depend on mitochondrial oxidative phosphorylation (OXPHOS) that produces ATP for muscle metabolism. We determined mitochondrial DNA (mtDNA) and ACTN3 genotypes in Finnish elite endurance ($n=52$) and sprint ($n=89$) athletes, and found that the frequencies of mtDNA haplogroups differed significantly between the two groups. Most notably, none of the endurance athletes belonged to haplogroup K or subhaplogroup J2, both of which have previously been associated with longevity. The frequency of ACTN3 XX genotype was higher and that of RR was lower among Finnish endurance athletes, and, in addition, none of the top Finnish sprinters had the XX genotype. Lack of mtDNA haplogroup K and subhaplogroup J2 among elite endurance athletes suggests that these haplogroups are 'uncoupling genomes'. Such genomes should not be beneficial to endurance-type athletic performance but should be beneficial to longevity, since uncoupling of OXPHOS reduces the production of ATP, reduces the release of reactive oxygen species and generates heat.
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Keywords: physical endurance; genetics; longevity

Introduction

Elite athletic performance is a complex trait, which displays a genetic trade-off between speed and endurance.[1] The I allele of angiotensin-converting enzyme (ACE), for example, has been associated with good endurance performance[2–4] and the D allele with good sprint performance.[5] Furthermore, the 577XX genotype of the ACTN3 (alpha-actinin 3) gene, leading to a loss of α-actinin-3 in fast-twitch muscle fibres, is more common among endurance athletes, whereas the wild-type 577RR genotype is more common among sprinters.[1] An association has also been suggested between good physical performance and polymorphisms in genes encoding proteins of mitochondrial energy metabolism. A polymorphism in the uncoupling protein-2 gene has been associated with good exercise efficiency,[6] and the 8794C>T transition in the MTATP6 gene, which determines mitochondrial DNA (mtDNA) haplogroup A, has been associated with good performance in endurance running.[7]

Aerobic ATP generation by oxidative phosphorylation (OXPHOS) in the mitochondrial respiratory chain is a prerequisite for prolonged muscle exercise. Out of >80 subunits of the respiratory chain enzymes, 13 are encoded by mtDNA that is a 16 568-bp maternally inherited genome. Interestingly, good aerobic performance has been found to be maternally inherited more often than paternally inherited.[8,9] Furthermore, patients with mutations in mtDNA commonly present with exercise intolerance, muscle weakness and increased production of lactic acid.[10]

An association has been found between several mtDNA control region polymorphisms and endurance capacity in

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sedentary men,[11] and between morph variants of MTND5 and the level of maximum oxygen uptake,[12] suggesting that certain mtDNA lineages may contribute to good aerobic performance. Endurance runners should have highly efficient ATP production by OXPHOS, whereas sprinters may rely more on anaerobic glycolysis. Despite the crucial role of mitochondrial function in endurance performance, and despite the recent data on lineage-specific differences in mtDNA,[13,14] differences in mtDNA haplogroups between sprinters and endurance runners have not previously been reported. We therefore set out to study mtDNA haplogroup and subhaplogroup frequencies in Finnish track and field athletes competing at the top level in endurance or sprint events. Furthermore, ACTN3 genotypes were assessed as a possible confounding variable.

Subjects and methods

Subjects

The subjects consisted of 141 Finnish elite track and field athletes (52 endurance athletes, 89 sprinters) including three pairs of twins (two among endurance athletes, one among sprinters) and five pairs of siblings (one among endurance athletes, four among the sprinters). Data on mtDNA haplogroup frequencies in 1060 Finnish control subjects were included in the analyses.[15,16] Athletes were included, if they had participated in the national track and field championships and/or in a national-level cross-country race. Their best achievements were requested and they were considered top athletes, if they had represented Finland in the European or World Track and Field Championships (20 endurance athletes, 23 sprinters). Endurance athletes were runners whose main event was a race between 800 m and a marathon, or walkers, and sprinters were athletes whose main event was a race between 100 and 400 m, or a field event. The birthplace of the maternal grandmother of the athletes was determined. Among the endurance athletes, 77% of the maternal grandmothers had been born south from the 64° latitude, and among the sprint athletes the corresponding figure was 82%. Furthermore, 81% of the controls had been born in this part of the country. These figures suggest that the groups were homogenous in their geographical background.

The study was approved by the Ethics Committee of Oulu University Hospital, and the athletes signed a statement of informed consent. The samples were collected from the athletes at the main national training camps with the permission of the Finnish Athletic Association.

Methods

MtDNA haplogroup and subhaplogroup analyses Total DNA was isolated from the blood cells using a QIAamp Blood Kit (Qiagen, Hilden, Germany). The mtDNA haplogroups and subhaplogroups were determined by restriction fragment analysis,[17,18] or, by direct sequencing if a restriction site was not available (Table 1). Sequence variation in the European population was used to identify the polymorphisms that determine the mtDNA subhaplogroups.[18,19] The allele status at position 8794 in MTATP6 was determined by direct sequencing.

ACTN3 genotyping ACTN3 genotypes have previously been found to differ between endurance and sprint athletes.[1] We determined these in order to assess whether they were a confounding variable in association analyses of mtDNA haplogroups. They were determined based on the C>T transition at nucleotide 1747 in exon 16, which leads to the replacement of an arginine codon by a stop codon.[21] Allele 577R (codon CGA) can be distinguished by the absence of a *Dde*I restriction site, and allele 577X (codon TGA) by its presence.[22] Digested PCR fragments were separated by 2% Metaphor® Agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) gel electrophoresis. In total, 40 (77%) of the endurance athletes and 68 (76%) of the sprinters, including one pair of twins, participated in the ACTN3 genotype analysis. Anonymous samples from 120 Finnish blood donors were used as controls.

Statistical analyses Exact test of population differentiation was used to assess differences in the frequencies of mtDNA haplogroups between the endurance and sprint athletes and to analyse differences in mtDNA haplogroup frequencies between the three ACTN3 genotypes.

Results

The frequencies of mtDNA haplogroups differed between the endurance and sprint athletes ($P=0.039$) (Table 2). Most notably, the frequencies of haplogroups J and K were higher among the sprinters. Only one endurance athlete belonged to haplogroup J (1.9%), whereas the frequency of this haplogroup among the sprinters was 6.7%. Two of the sprinters belonged to subhaplogroup J2, while the remaining four sprinters and the endurance athlete belonged to subhaplogroup J1. None of the endurance athletes belonged to haplogroup K, whereas the frequency of this haplogroup among the sprinters was 9.0%. In addition, four endurance athletes (6.6%) belonged to haplogroup I, but none of the sprinters. The frequencies of mtDNA haplogroup clusters (HV, KU, IWX, JT) did not differ significantly between the groups. None of the Finnish athletes harboured 8794C>T in MTATP6.

The possibility of recent common maternal ancestors was examined by using information on the birthplaces of the maternal grandmothers of the subjects. We identified only two pairs of cases with identical mtDNA subhaplogroup and with the same city of origin of the grandmother. In both cases, the subjects belonged to

Table 1 Polymorphisms used to determine mtDNA haplogroups and subhaplogroups

Haplogroup/ subhaplogroup	*Nucleotide variant*	*Restriction enzyme*
H	7028C	*−AluI*
H1	3010G>A	*−Hpy188III*
H2	4769A	*+AluI*
H3	6776T>C	NA
V	4580G>A	*−Hsp92II*
V1	5263C>T	*−HaeIII*
U	12308A>G	*+DdeI*[a]
U2	3720A>G	*+BseNI*
U5	9477G>A	*+Tsp509I*
U8	9698T>C	NA
K	12308A>G	*+DdeI*[a]
	10398A>G	*+DdeI*
*K1**	9093A>G	NA
T	15607A>G	*+AluI*
T1	12633C>A	*−AvaII*
T2	11812A>G	NA
J	13708G>A	*−MvaI*
J1	3010G>A	*−Hpy188III*
J2	7476C>T	*−AluI*
W	8251G>A	*+AvaII*
W1	12669C>T	*+NdeII*
W2	9612G>A	NA
I	1719G>A	*−DdeI*
	8251G>A	*+AvaII*
I1	6734G>A	*−EcoRV*
I2	15758A>G	*+DdeI*
X	1719G>A	*−DdeI*
Z	10400C>T	*+AluI*

The haplogroups and subhaplogroups were determined by restriction fragment analysis, or by direct sequencing when no restriction site was available. Subhaplogroup K1 is defined by 1189T>C,[19] and 9093A>G defines a subset of this subhaplogroup which appears to be fairly frequent among the Finns.[18] Allele status at a variable site is reported relative to rCRS. + = gain of restriction site, − = loss of restriction site, NA = restriction site is not available.
[a]Restriction site created by use of a mismatched oligonucleotide.[20]

subhaplogroup H1. This subhaplogroup is frequent among the Finns suggesting that the possibility of a recent common maternal ancestor is low.

We found that the frequency of the 577XX genotype of ACTN3 was lower and that of 577RR higher among the sprinters than among the endurance runners, and when the groups were stratified into subsets on the basis of their best achievements, there was an inverse correlation ($P = 0.03$) between the frequency of 577XX and success in sprinting events (Figure 1). Furthermore, none of the top sprinters harboured the 577XX genotype. There were no differences in mtDNA haplogroup frequencies between the three ACTN3 genotypes ($P = 0.98$; exact test of population differentiation).

Discussion

We found that the frequencies of mtDNA haplogroups differed significantly between Finnish endurance and sprint athletes. None of the endurance athletes belonged to either subhaplogroup J2 or haplogroup K, whereas the combined frequency of these was 11.2% among the sprinters. The frequency of subhaplogroup J2 in the Finnish population is at least 1.5% and that of haplogroup K 3.0% making the combined frequency of these haplogroups among the Finns 4.5%.[15] Both haplogroup J and haplogroup K are minor in European populations and, therefore, small changes in the frequencies of the cases within study groups may abolish the significance of the statistical testing. Interestingly, however, an association has been found between haplogroup J and longevity[16,24,25] and subhaplogroup J2 in particular is more frequent among the very old than among controls.[16] Haplogroup K has also been associated with longevity,[25,26] or shows a trend for increased frequency in the very old.[16,24] Thus, the subhaplogroups that are found at an increased frequency among the very old are rare among endurance athletes suggesting complementarities between these data.

We found that the 577XX genotype of the ACTN3 gene was less common among Finnish sprinters and that none of the top sprinters harboured this genotype. A trade-off between endurance and sprint performance traits seems possible, since the 577XX genotype has been found at a higher frequency among endurance athletes than among sprinters, while none of the most successful sprinters has been found to harbour this genotype.[1] These differences in the distribution of the ACTN3 genotypes did not, however, explain our finding that mtDNA haplogroups differ between the two groups of athletes. The 577XX genotype of ACTN3 is present in approximately 16% of the world's population,[21] and although it causes complete absence of α-actinin-3 in fast-twitch skeletal muscle fibres, it is not associated with any clinical phenotype.[21,22]

Endurance athletes should be a highly selected group in terms of efficiency of ATP production. The main function of the mitochondria is to produce ATP by OXPHOS, and while the uncoupling of OXPHOS generates heat, it concomitantly reduces the production of ATP due to decreased proton translocation across the mitochondrial inner membrane or due to proton leak via ATP synthase.[27] Uncoupling of OXPHOS also lowers the production of reactive oxygen species (ROS), which are its oligatory by-products.[27,28] ROS may play a role in ageing,[29,30] for interestingly, mice with enhanced mitochondrial uncoupling live longer[31] and mice with deficient

Table 2 Mitochondrial DNA haplogroup and subhaplogroup frequencies among the endurance and sprint athletes

Haplogroup	Endurance (n = 52)		Sprint (n = 89)		Controls (n = 1060)	
	n	%	n	%	n	%
H	27	52	42	47	508	48
H1	13	25	22	25	ND	ND
H2	3	5.8	2	2.2	ND	ND
H3	1	1.9	3	3.4	ND	ND
H*	10	19	15	17	ND	ND
V[a]	3	5.8	7	7.9	51	4.8
V1	0	0	2	2.2	ND	ND
V*	2	3.8	5	5.6	ND	ND
Pre-V	1	1.9	0	0	ND	ND
U	11	21	13	15	253	24
U5	9	17	13	15	ND	ND
U8	1	1.9	0	0	ND	ND
U*	1	1.9	0	0	ND	ND
K	0	0	8	9.0	48	4.5
K1[b]	0	0	5	5.6	ND	ND
K*	0	0	3	3.4	ND	ND
T	3	5.8	4	4.5	38	3.6
T1	2	3.8	2	2.2	ND	ND
T2	1	1.9	2	2.2	ND	ND
J	1	1.9	6	6.7	51	4.8
J1	1	1.9	4	4.5	ND	ND
J2	0	0	2	2.2	ND	ND
W	3	5.8	6	6.7	47	4.4
W1	1	1.9	3	3.4	ND	ND
W2	2	3.8	1	1.1	ND	ND
W*	0	0	2	2.2	ND	ND
I	4	7.7	0	0	30	2.8
I1	2	3.8	0	0	ND	ND
I2	2	3.8	0	0	ND	ND
X	0	0	2	2.3	12	1.1
Others	0	0	1	1.1	22	2.1

Samples that did not fall into any of the subhaplogroups shown are denoted by an asterisk (*).
[a]One endurance athlete belonged to haplogroup pre-V, harbouring 7028T (+7025*AluI*), 4580G (+4577Hsp92II), 14766C (−14766*MseI*) and 15904C (−15904*MseI*) in the coding region and 16298C and 16362C in HVS-I.[23]
[b]K1 is a subhaplogroup of haplogroup K defined by 9093A>G.[18,19]
Frequencies of mtDNA haplogroups among 1060 Finnish subjects are shown as controls.[15,16] ND = not determined.

uncoupling produce more ROS.[32] An mtDNA genotype leading to less efficient OXPHOS and lower ATP production (an 'uncoupling genotype') would therefore produce less ROS as well[33] and probably promote longevity. MtDNA polymorphisms that increase uncoupling and concomitant heat production may have been important in climatic adaptation, and may therefore have been subject to positive selection.[14,34] Indeed, neutrality tests have

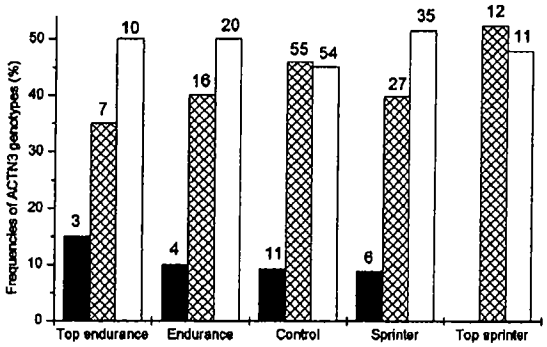

Figure 1 Frequencies of ACTN3 genotypes in Finnish track and field athletes and population controls. The endurance athletes and sprinters were both stratified into two subsets according to their best achievements. Solid bar, 577XX genotype; open bar, 577RR genotype; cross-hatched bar, 577RX genotype. Number of cases is shown above the bars.

revealed lineage-specific differences between the various European mtDNA haplogroups and haplogroup clusters, suggesting that mtDNA has evolved in a lineage-specific manner.[13]

We suggest that subhaplogroup J2 and haplogroup K are 'uncoupling genomes.' There are no previous studies on biochemical properties of cells harbouring different mtDNA lineages, but, interestingly, sperm cells harbouring mtDNA haplogroup T have been found to be slower in motility than cells harbouring haplogroup H.[35] This finding suggests that mtDNA lineages may differ in their functional properties. Functional or biochemical tests should reveal that a respiratory chain encoded by 'an uncoupling genome' produces less ROS and less ATP. Due to uncoupling, these genomes, such as haplogroup K and subhaplogroup J2, promote longevity[16,24–26] but are not favourable in situations, where highly efficient ATP production is required, such as endurance athletic performance.

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The *ACTN3* Gene in Elite Greek Track and Field Athletes

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- gene
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- alpha-actinin-3

Abstract

The study of genetic influence in the making of an Olympic champion is still in its nascence, but recent work has provided findings regarding the association of the *ACTN3* gene on athletic performance. The aim of this study was to examine genetic differences among elite Greek track and field athletes by analysing a mononucleotide polymorphism in exon 15 of the *ACTN3* gene. Results showed that *ACTN3* genotype and allele frequencies in the top power-oriented athletes were statistically significantly different from those in a representative random sample of the Greek population: the frequency of the RR *ACTN3* genotype in power-oriented athletes vs. the general population was 47.94% vs. 25.97%. This result was even more prominent for comparison of the subgroup of sprinters to controls. The results suggest an overall strong association between the presence of the RR genotype and elite power performance.

Introduction

Only a few attempts have been made to shed light upon the influence of genes in making an Olympic champion [7]. The first evidence that a mononucleotide difference in DNA sequence was associated with power ability referred to the R577X polymorphism of the *ACTN3* gene [12]. A transition (C > T) at nucleotide position 1747 in the *ACTN3* coding sequence converts an arginine (R) to a stop codon (X) at residue 577 [5], and this change results in the complete loss of α-actinin-3 protein function in homozygous XX individuals [12]. Alpha-actinin-3 is the most-highly specialised of the four mammalian alpha-actinins, with its expression restricted to type II (fast-twitch) myofibres in skeletal muscle [3,5]. Although it is likely that α-actinin-3 protein has many similar roles to α-actinin-2 [3,5], there is strong evidence to suggest that *ACTN3* gene has been maintained in the genome because of function(s) independent of *ACTN2* [12].

The RR genotype of the *ACTN3* gene has been found to be associated with elite performance in activities requiring bursts of strength in combination with speed (power oriented activities) [6, 12]. The XX *ACTN3* genotype occurs at a higher frequency in some cohorts of elite endurance athletes [2,6]. Additionally, a statistically significant difference between endurance athletes and controls was found in females [12], raising the possibility that the total deficiency of the α-actinin-3 protein may confer some beneficial effect on endurance performance. In contrast to the studies mentioned above, there has been recent evidence that an XX *ACTN3* genotype in elite Ethiopians and Kenyan long-distance runners is not associated with endurance performance [13]. To try to further clarify what relevance *ACTN3* polymorphisms might have to performance, we examined their frequency in elite Greek track and field athletes covering a spectrum from power-oriented short distances to endurance-based longer distances. Greeks are southern European, whereas the other two studies are of northern Europeans [6,12]. Thus, studying Greeks gives a more complete picture of European athletes, as European is a generic term for a very heterogeneous population. Therefore, the aim of our study is to elucidate the genetic differences among a group of elite Greek power-oriented track and field athletes and a random representative sample of the Greek population.

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Table 1 The % of Olympic, European and Balkan level athletes for power oriented and endurance athletes group

Athlete groups	Olympic level	European level	Balkan level	Total
Power-oriented	34%	26%	40%	n = 73
Endurance-oriented	36%	14%	50%	n = 28

Materials and Methods

Subjects and controls

The subjects consisted of 101 elite Greek track and field athletes (73 males and 28 females). Athletes were defined as elite and included in the sample if they had been chosen to represent Greece at the international level. Achievements among our sample group included world, European and Balkan records (⚙ Table 1), and many of the athletes were Olympic medallists or finalists and world or European champions. The power-oriented top-level track and field athletes group included 34 sprinters (whose main event was a race of 100 to 400 m), 23 jumpers, 9 throwers and 7 decathletes. The endurance athletes group included 19 long distance runners (whose main event was a race ranging from 3000 m to a marathon length), 4 middle distance runners (whose main event was a race ranging from 800 m to 1500 m length), 3 triathletes (top performers in distance running races) and 2 walkers. The representative random control group was 181 unrelated, healthy, Greek individuals. The birthplaces of the athletes and of the controls were matching in their geographical distribution. All subjects had the Greek nationality and were Caucasians. The study protocol was in accordance with the ethical and moral procedures of the Aristotle University Research Committee.

Genotyping

Blood samples were obtained from all individuals and DNA was isolated from white blood cells by a standard protocol [4]. The 291 bp fragment of exon 15 of the *ACTN3* gene was amplified by PCR using the forward primer 5′-CTGTTGCCTGTGGTAAGTGGG-3′ and the reverse primer 5′-TGGTCACAGTATGCAGGAGGG-3′ as recommended by Mills et al. [5]. PCR reaction mix contained 1 µl (30–50 ng) DNA, 0.2 µl primers (100 pmol/µl), and 0.15 µl Taq Polymerase (5 Units/µl). PCR was performed for 30 cycles (30 s each of denaturation at 94 °C, annealing at 58 °C, and extension at 72 °C).

The amplified PCR fragments were subsequently digested with *Dde*I endonuclease (New England Biolabs, Beverly, MA, USA), and the alleles 577R and 577X were distinguished by the presence (577X) or absence (577R) of a *Dde*I restriction site. Digestion of PCR products of the 577X allele yields bands of 108, 97 and 86 bp, whereas digestion of PCR products of the 577R allele yields bands of 205 and 86 bp. Digested products were then electrophoresed in 5% polyacrylamide gels and silver stained.

Statistical analysis

Genotype and allele frequencies were compared between elite athletes and controls by the chi-squared test using the statistical package GENEPOP V. 3.4 (updated version of GENEPOP V1.2) [8]. Tests for the HW equilibrium were also done with the same package.

Table 2 *ACTN3* genotype and allele frequencies in 101 elite Greek track and field athletes and 181 control individuals

Group (n)	Genotype (%)			Allele frequency (%)	
	RR	RX	XX	R	X
°Power athletes (73)	47.94	35.62	16.44**	65.8	34.2**
Sprinters (34)	73.53	17.65	8.82[‡]	82.4	17.6[‡]
Endurance athletes (28)	50	25	25	62.5	37.5
Controls (181)	25.97	55.80	18.23	53.9	46.1

** $p < 0.02$. [‡] $p < 0.001$ comparison with controls; ° Power athletes include the 34 sprinters

Results

A total of 101 Greek elite athletes and 181 control individuals were analysed in this study. ⚙ Table 2 shows the *ACTN3* genotype and allele frequencies from the 73 power-oriented athletes (including 34 sprinters), 28 endurance athletes, and the control group. The observed genotype counts for the power-oriented athlete group as a whole and the control group were not statistically different from those expected under Hardy-Weinberg equilibrium. However, absence of HW equilibrium was found for the small groups of sprinters (extracted from the larger group of power-oriented athletes) and for endurance athletes.

The distribution of the genotypes showed differences among the various groups (⚙ Table 2, Fig. 1). There were statistically significant differences in the frequencies of alleles ($p = 0.017$) and genotypes ($p = 0.016$) between elite power-oriented athletes and the control group: the power-oriented athletes displayed a lesser frequency of the X allele than the control group. Statistically significant differences were even more prominent when comparing the allele frequencies ($p = 0.0001$) and genotype frequencies ($p = 0.0001$) of the subgroup of sprinters to the control group. It should be noted that all of the Olympic/European-level sprinters in the study sample had at least one R allele for *ACTN3* (⚙ Fig. 1). As compared to the control group, sprinters had a lower frequency of the XX genotype (8.82% vs. 18.23%), and higher frequency of the RR genotype (73.53% vs. 25.97%) ($p < 0.0001$). In contrast, there was a trend of increased frequency of the XX genotype in the elite endurance athletes group, as compared to sprinters and to the control group (⚙ Table 2). However, there were no significant differences in the frequencies of alleles ($p = 0.252$) or genotypes ($p = 0.238$) between the endurance athletes group and the control group or between the power-oriented athletes group and the endurance athletes group for allele ($p = 0.742$) and genotype ($p = 0.771$) comparisons.

Discussion

The current study of elite athletic performance by means of a molecular genetic marker suggests that the presence of α-actinin-3 protein has a positive effect on power performance, a finding consistent with structural and signalling functions of α-actinin-3 protein in fast-twitch muscle fibres.

Yang et al. [12] genotyped 107 power-oriented Australian athletes involved in a wide range of sports (including 46 track and field athletes) and found that none of those who had competed at the Olympic level (n = 32) had an XX genotype (α-actinin-3 deficient). Additionally, in a group of 89 Finnish power-oriented track

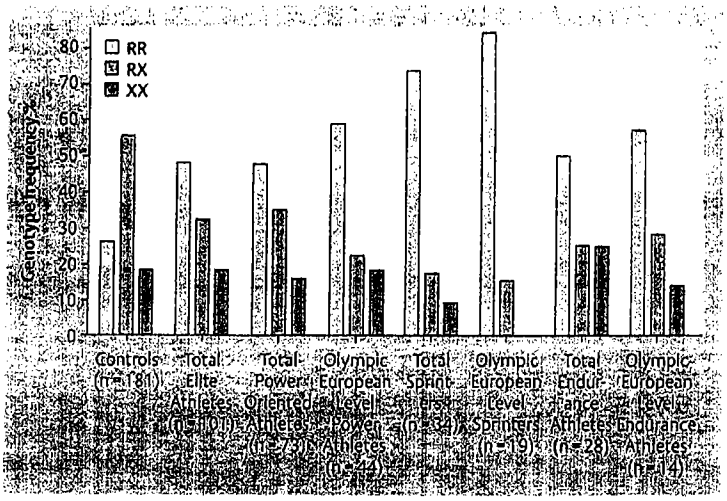

Fig. 1 Frequencies of the *ACTN3* genotypes in 101 elite Greek track and field athletes and 181 control individuals.

and field athletes who competed at the national level, Niemi et al. [6] similarly found that none of these athletes (n = 23) had an XX genotype. In our study of a cohort of 73 Greek power-oriented track and field athletes (including 34 sprinters) who competed at the international level, none of the sprinters who had represented Greece in the Olympic Games or in the world or European championships (n = 19) had an XX genotype (◘ **Fig. 1**). Thus, *ACTN3* is the first skeletal-muscle gene for which such an association has been demonstrated in three different population groups (Australian, Finnish, and Greek) of athletes competing at the Olympic, world, and European level (total sample size: 32 + 23 + 19 = 69). These findings, in conjunction with the statistically significant differences between the power-oriented athletes groups and controls that were demonstrated in all of the above studies, suggest that the R allele of *ACTN3* gene is an advantageous determinant for achieving high-level sprint-power performance.

In Greek sprinters, the X allele of *ACTN3* was notably less frequent and the R allele was more frequent than in endurance runners (◘ **Table 2**). This "gene trade-off" was also evident in Australian athletes (power-oriented vs. endurance) [12]. The above data support the hypothesis that the performance constraint, in which decathletes are called upon to perform at high levels in both sprint (100 m) and endurance (1500 m) races [11], may have a genetic basis. These results also show that the frequency of the RR genotype is higher not only when higher-performing athletes are genotyped, but also when a more specific athletic phenotype (running ability) is analysed, suggesting that the phenotypic homogeneity of the study sample (i.e., studying only track and field athletes or spinters) increases the efficiency of finding candidate genes involved in top human performance. This study demonstrates that sprinting ability is primarily affected by the RR genotype, possibly because sprinting is less influenced by anthropometric and technique factors compared to other sports. In line with this hypothesis and together with the fact that some of the top Olympic/European level athletes (5 high jumpers, 2 decathletes and 1 thrower) in our cohort were XX, it seems possible that success in more complex sports is less influenced by the RR genotype.

A study examining the genotype of East African endurance runners found that although the success of this group is possibly attributable to their genes, surprisingly, only 1% of elite Kenyan runners had an XX *ACTN3* genotype [13]. Our results revealed a trend towards a higher frequency of the XX genotype in the overall group of endurance athletes, but not in the more elite subset of Olympic/European-level endurance athletes (◘ **Fig. 1**). The frequency of the RR genotype among 14 Olympic/European-level endurance runners in our study was high (57%) and some of Greek world's top-level marathon competitors had an RR genotype. This data supports the hypothesis of Lucia et al. [2], that top-level endurance runners require not only slow-twitch myofibres, but also the ability to recruit type II fast-twitch myofibres during the competitively critical phases of an endurance race in which they must effectively sprint for short distances.

However, in all studies of endurance Caucasians athletes, the XX genotype predominates. Yang et al. [12] found a higher frequency of the XX genotype in a study of 107 white Australian endurance athletes. Additional studies of white professional endurance cyclists of European ancestry [12] and of national-level Finnish endurance athletes [6] in concert, show a higher frequency of the XX genotype in our overall endurance-athlete cohort, which included the elite subset of Olympic-level endurance athletes. Data from studies of other Caucasian cohorts [2,6,12] and the relatively low X allele frequencies found in Europeans [12] form a basis for the hypothesis that the X allele confers an advantage in endurance performance, although the mechanism(s) by which this occurs is not clear and may involve uncharacterised gene-gene interactions.

Based on our results and on recent evidence, we suggest the following: It seems that the α-actinin-3 protein does not have an inhibitory effect in aerobic metabolism, because many Caucasians [2], 99% of Kenyan elite endurance athletes [13], and 75% of Greek international-level competitors in endurance-based sports had at least one R allele of the *ACTN3* gene. The beneficial effect of α-actinin-3 deficiency on endurance performance is less important than its presence on sprinting-power performance, as four different studies showed that none of the Olympic-level sprinters examined had an α-actinin-3 deficient XX genotype, but many of the Olympic-level endurance athletes analysed had an RR genotype.

Very little is known about the specific genotypes that contribute to individual responses to training. According to the training principle of individuality, each athlete responds differently to the same quantitative and qualitative training stimuli [10], leading to a range of responses among athletes who have experienced otherwise identical training programmes. The influence of the *ACTN3* R577X polymorphism on each athlete's response to training needs to be more closely examined to explain whether this polymorphism defines the athlete's initial levels of power ability or it actually affects the response to training. Interestingly, Roth et al. [9] recently found that the RR genotype is associated with muscle power response to training and Clarkson et al. [1] showed that XX homozygotes had the lowest baseline strength. Thus, although further investigation is needed to fully understand the function of the *ACTN3* gene, it is evident that *ACTN3* polymorphisms can influence an athlete's power-sprint performance, and we are currently undertaking further genetic studies to investigate this.

The results of this study and of others show that the R allele of *ACTN3* gene is an advantageous allele, in the Olympic-level of sprinting performance, while the X allele may confer some benefit to endurance performance in Caucasians, but is not a determinant for their success in endurance-based sports. Taken together, these results suggest that the *ACTN3* gene can be used as a molecular genetic marker to at least partially predict an athlete's ability to achieve peak power and sprinting performance.

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ACTN3 R577X Nonsense Allele (X) is Under-Represented in Elite-Level Strength Athletes: 1722: *Board #10* May 31 8:00 AM 9:30 AM

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PURPOSE: Alpha-actinin-3 is a structural protein of the sarcomeric Z-disc that is specifically expressed in skeletal muscle Type II muscle fibers. A common nonsense polymorphism in codon 577 of the ACTN3 gene (R577X) results in alpha-actinin-3 deficiency in X-allele homozygotes (X/X genotype). Alpha-actinin-3 deficiency has been reported in ~18% of healthy white individuals and ~6% of healthy black (African American) individuals. Previous reports have shown a lower proportion of sprint/power related athletes carry the ACTN3 X/X genotype than is expected compared to the general population (Yang et al., 2003; Niemi and Majamaa, 2005), possibly attributed to impairment of muscle function related to the alpha-actinin-3 deficiency. We have also observed lower muscle mass in X/X females (Walsh et al., MSSE abstract #873, 2005). In the present study, we examined the frequency of the X/X genotype in both black and white elite bodybuilders and strength athletes in comparison to the general population. We hypothesized that fewer X-allele homozygotes would be identified within the strength athletes compared to the general population.

METHODS: A reference population of 668 whites (374 men and 294 women) and 208 blacks (106 men and 102 women) from the Baltimore Longitudinal Study of Aging were genotyped for the ACTN3 R577X polymorphism. Strength athletes (52 white and 23 black; 4 women) consisting predominantly of world-class bodybuilders (n=18 ranked in the top 100 worldwide), locally competitive bodybuilders, and elite power lifters were recruited and similarly genotyped.

RESULTS: Consistent with previous reports, the frequency of the X/X genotype was 19.9% in the white subjects and 4.8% in the black subjects of the reference population. In contrast, the X/X genotype frequency was only 9.6% in the white strength athletes (P=0.035, Chi square analysis) and 0% in the black strength athletes (not significant, P=0.21, owing to sample size limitations).

CONCLUSIONS: The results indicate that the ACTN3 R577X nonsense allele (X) is under-represented in elite strength athletes, consistent with previous reports suggesting the possibility that alpha-actinin-3 deficiency impairs muscle performance.

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Loss of *ACTN3* gene function alters mouse muscle metabolism and shows evidence of positive selection in humans

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More than a billion humans worldwide are predicted to be completely deficient in the fast skeletal muscle fiber protein α-actinin-3 owing to homozygosity for a premature stop codon polymorphism, R577X, in the *ACTN3* gene. The R577X polymorphism is associated with elite athlete status and human muscle performance, suggesting that α-actinin-3 deficiency influences the function of fast muscle fibers. Here we show that loss of α-actinin-3 expression in a knockout mouse model results in a shift in muscle metabolism toward the more efficient aerobic pathway and an increase in intrinsic endurance performance. In addition, we demonstrate that the genomic region surrounding the 577X null allele shows low levels of genetic variation and recombination in individuals of European and East Asian descent, consistent with strong, recent positive selection. We propose that the 577X allele has been positively selected in some human populations owing to its effect on skeletal muscle metabolism.

The α-actinins are an ancient family of actin-binding proteins. Of the four mammalian α-actinins, α-actinin-3 is the most specialized, with expression restricted largely to the fast glycolytic skeletal muscle fibers[1] that are responsible for rapid force generation. Notably, α-actinin-3 is absent in ~18% of healthy individuals of European descent owing to homozygosity for a common nonsense polymorphism in the *ACTN3* gene, R577X[2]. The frequency of the 577X null allele differs between human groups: it is ~10% in Africans but approaches 50% in Eurasian populations[1]. Two independent studies have reported associations between R577X and elite athlete status[3,4]; the frequency of the 577XX null genotype is lower in sprinting and power athletes and higher in endurance athletes. The 577XX genotype has also been associated with lower muscle strength[5] and poorer sprint performance[6] in two large studies of non-athletes. The mechanism(s) by which α-actinin-3 deficiency influences muscle function remain unclear.

To characterize the phenotypic consequences of α-actinin-3 deficiency, we have generated an *Actn3* knockout mouse line (**Fig. 1a**). Homozygous knockout ($Actn3^{-/-}$) mice do not express any detectable α-actinin-3 protein, as assessed by either immunohistochemistry or immunoblotting (**Fig. 1b,c**). $Actn3^{-/-}$ mice were morphologically similar to wild-type ($Actn3^{+/+}$) littermates, they showed normal sarcomere formation at the light and electron microscope level and they did not demonstrate substantial loss of fast glycolytic (2B) fibers (data not shown). Loss of α-actinin-3 in fast fibers seems to be compensated for by an upregulation of the related protein α-actinin-2 (**Fig. 1c**), which shifts from preferential expression in oxidative fibers to uniform staining in all fibers in $Actn3^{-/-}$ muscle (**Fig. 1b**). The $Actn3^{-/-}$ mouse thus mimics α-actinin expression in 577XX humans, who also show α-actinin-3 deficiency and α-actinin-2 expression in all muscle fibers[1].

Given the differing frequencies of the 577XX genotype in elite sprint and endurance athletes, we investigated whether the loss of α-actinin-3 altered skeletal muscle metabolism. We found that muscle sections from $Actn3^{-/-}$ mice showed more intense staining than those from $Actn3^{+/+}$ mice for two markers of aerobic metabolism, NADH-tetrazolium reductase (NADH-TR) and succinate dehydrogenase (SDH) (**Fig. 2a**). Quantitative analysis showed a significant ($P < 0.02$) increase in regions staining positively for NADH-TR in $Actn3^{-/-}$ muscle compared to $Actn3^{+/+}$ muscle. Immunohistochemical analysis with myosin heavy chain 2B, a marker of fast glycolytic fibers, indicated that the increase in oxidative enzyme activity does not result from a loss of fast fibers but rather from altered metabolism in these fibers (**Fig. 2b**). Two mitochondrial markers (cytochrome c oxidase and

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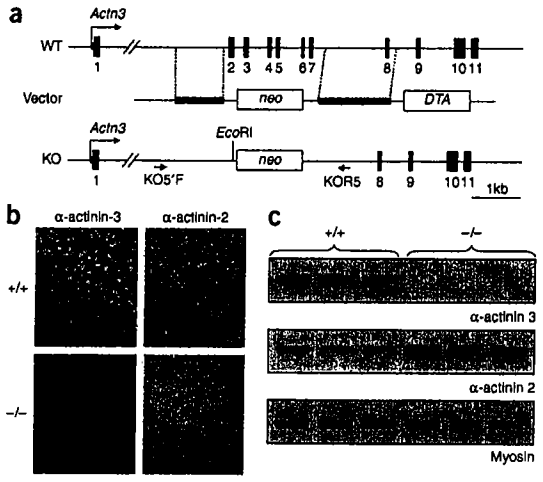

Figure 1 Generation and analysis of *Actn3*$^{-/-}$ mice. (a) Generation of the *Actn3* knockout locus. Homologous recombination in *Actn3*$^{+/+}$ embryonic stem cells results in replacement of *Actn3* exons 2–7 with the *neo* marker. (b) Immunohistochemistry for the sarcomeric α-actinins in *Actn3*$^{+/+}$ and *Actn3*$^{-/-}$ mice, showing loss of α-actinin-3 and altered expression pattern of α-actinin-2 in *Actn3*$^{-/-}$ muscle. (c) Immunoblot analysis shows absent α-actinin-3 and upregulated α-actinin-2 in *Actn3*$^{-/-}$ mice compared to *Actn3*$^{+/+}$ littermates. Total myosin is shown as a loading control.

mitochondrial porin) were upregulated in the muscle of *Actn3*$^{-/-}$ mice (**Fig. 3a**), consistent with increased mitochondrial density in *Actn3*$^{-/-}$ muscle.

As further confirmation of these metabolic findings, we analyzed the activity of two key enzymes involved in pyruvate metabolism (**Fig. 3b**). Pyruvate, the end metabolite of the glycolytic pathway, represents a branch point in muscle metabolism: in fast muscle fibers it is predominantly converted to lactate by the anaerobic pathway enzyme lactate dehydrogenase (LDH), and in slow fibers it is preferentially oxidized within the mitochondria, a slower but more efficient process in which citrate synthase catalyses the first reaction. The activity of LDH was 16% lower ($P < 0.001$) and that of citrate synthase was 22% higher ($P < 0.05$) in *Actn3*$^{-/-}$ mice compared to *Actn3*$^{+/+}$ controls. These results indicate that the loss of α-actinin-3 results in a significant shift in the metabolic pathways of fast muscle fibers away from their typical reliance on the anaerobic lactate pathway toward the slower but more efficient aerobic pathway normally associated with slow muscle fibers.

To determine if this alteration in skeletal muscle metabolism influences endurance performance, we subjected *Actn3*$^{-/-}$ mice and *Actn3*$^{+/+}$ littermate controls to a modified version of the intrinsic exercise capacity test, in which mice were run on a motorized treadmill at increasing speeds until they reached exhaustion. *Actn3*$^{-/-}$ mice performed significantly better than *Actn3*$^{+/+}$ mice in this test, running on average 33% further than their *Actn3*$^{+/+}$ littermates before reaching exhaustion (**Fig. 3c**). These findings suggest that the shift toward oxidative metabolism observed in the muscle of *Actn3*$^{-/-}$ mice increases intrinsic endurance performance, consistent with the overrepresentation of the 577XX null genotype in elite female endurance athletes[3].

The phenotypic changes associated with α-actinin-3 deficiency in mice, coupled with the association of the 577X allele with reduced sprint and increased endurance performance, suggest that the 577X allele also influences muscle metabolism in humans. This in turn raises the possibility that the high frequency of 577X in some human populations has resulted from historical natural selection for increased metabolic efficiency. To investigate this possibility, we sequenced a 3.7-kb region of genomic DNA, centered on R577X, in 96 DNA samples previously analyzed by the International HapMap Project[7] (**Supplementary Table 1** online), using primers described in **Supplementary Table 2** online. Our cohort included 32 Utah residents with ancestry from northern and western Europe (CEU), 32 Yoruba from Ibadan, Nigeria (YRI), 16 Han Chinese from Beijing (CHB) and 16 Japanese from Tokyo (JPT). The 32 CHB and JPT samples were grouped into a single cohort, designated ASN. For comparison, this region was also sequenced from a single chimpanzee.

Within our cohorts, we found frequencies of the 577X null allele similar to those reported previously[1]: 0.55 in CEU, 0.52 in ASN and 0.09 in the African YRI cohort. Owing to the low population frequency of the 577X allele in the YRI cohort, we restricted further analyses to the 64 Eurasian (CEU+ASN) samples. The sequenced region in the CEU+ASN cohorts contains 21 SNPs, including five missense polymorphisms and the R577X nonsense SNP, and one three base-pair indel (**Supplementary Table 3** online). These polymorphisms resolve into 12 haplotypes in three major haplogroups (**Fig. 4a**) of which two (R1 and R2) carry the 577R allele, and the third (X) carries the 577X allele. A long branch separates the X haplogroup from the relatively closely related R1 and R2 clusters. An excess of nonsynonymous substitutions associated with this branch is consistent with relaxed selective constraint specific to the lineage carrying the 577X alteration.

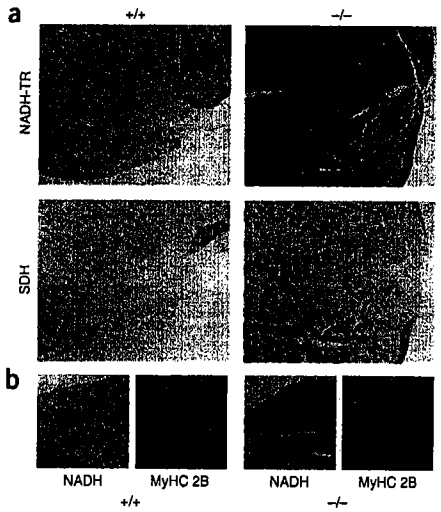

Figure 2 Fast muscle fibers in *Actn3*$^{-/-}$ mice show increased staining for markers of aerobic metabolism. (a) Representative images of quadriceps muscle sections show increased staining for NADH-tetrazolium reductase (NADH-TR) and succinate dehydrogenase (SDH) in *Actn3*$^{-/-}$ muscle compared to *Actn3*$^{+/+}$ mice. (b) Higher-power images of serial sections stained for myosin heavy chain (MyHC) 2B and NADH show that fast glycolytic (2B-positive) fibers stain more darkly for NADH-TR in *Actn3*$^{-/-}$ muscle.

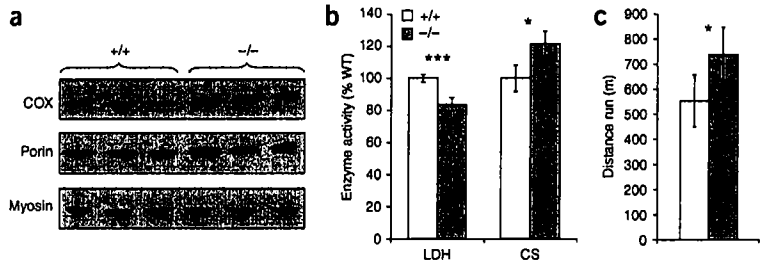

Figure 3 *Actn3*$^{-/-}$ muscle shows increased expression of mitochondrial markers, altered metabolic enzyme activity and increased endurance capacity. (a) Immunoblot analysis shows increased expression of the mitochondrial markers cytochrome c oxidase (COX) and mitochondrial porin in *Actn3*$^{-/-}$ mice compared to *Actn3*$^{+/+}$ littermates. Total myosin is shown as a loading control. (b) *Actn3*$^{-/-}$ mice show decreased lactate dehydrogenase (LDH) activity and increased citrate synthase (CS) activity compared to *Actn3*$^{+/+}$ (WT) littermates. Data were collected from seven *Actn3*$^{+/+}$ and seven *Actn3*$^{-/-}$ muscle lysates. For each enzyme, mean *Actn3*$^{+/+}$ activity is set to 100%. (c) Total distance run before exhaustion in an intrinsic exercise capacity test by 20 *Actn3*$^{+/+}$ and 23 *Actn3*$^{-/-}$ mice. In b and c, error bars indicate 95% c.i. * $P < 0.05$, *** $P < 0.001$.

Although this increased substitution rate makes precise dating difficult, the length of the branch separating 577X haplotypes from the inferred ancestral sequence and the presence of the 577X allele in all studied human populations[1] suggest the 577X substitution preceded the appearance of anatomically modern humans in Europe and Asia 40,000–60,000 years ago (40–60 kya). Notably, despite the apparent age, high frequency and relaxed selective constraint associated with the 577X allele, the 577X haplogroup contains little variation. In the CEU sample, we did not observe any variation among 35 577X haplotypes, and in the ASN cohort, we observed only three low-frequency polymorphisms on 5/33 577X haplotypes. This low level of variation suggests that the 577X allele achieved its current Eurasian frequency of ~0.5 in a comparatively recent and rapid expansion.

To determine whether this expansion was driven by positive natural selection, we examined the pattern of long-range linkage disequilibrium (LD) surrounding R577X using SNP genotype data from the HapMap project[7]. We employed a conservative algorithmic approach (see Methods) to determine the length of the unbroken long-range haplotype carrying a particular allele, which we refer to as the length of complete homozygosity (LCH). Recent positive selection should result in a long LCH associated with the selected allele, relative to allele frequency and local recombination rate.

We determined the LCH for a genome-wide set of >17,000 autosomal SNP alleles matched to 577X for both allele frequency and local recombination rate in the CEU and ASN HapMap cohorts. Demographic forces should affect LD at all autosomal loci equally, whereas positive selection will result in unusually large locus-specific values of long-range LD[8]. In the CEU cohort, the haplotype carrying the 577X allele is associated with a larger LCH (338,148 bp; **Fig. 4b**) than any other region in the comparison set: more than 100 kb longer than the next-largest value, and more than 100 times the median (3,253 bp) (**Fig. 4c**). The LCH for 577X in the ASN cohort (97,927 bp) is less extreme but is still longer than 98.9% of the values in the comparison set. The inferred recombination rate for the 577R allele in this region is substantial (**Fig. 4b**), demonstrating that the extended haplotypes carrying the 577X allele are not simply an artifact of low local recombination rate. The outlier status of the 577X allele in both populations strongly suggests that the haplotype carrying this allele has been subjected to recent positive selection in European and Asian populations.

The LCH in the CEU and ASN cohorts are both asymmetrical with respect to R577X, but they extend in opposite directions (**Fig. 4b**), probably owing to stochastic recombination events during the expansion of the selected haplotype in the two populations. The LCH regions contiguous with R577X overlap for only ~29.5 kb, spanning two genes: *ACTN3* and *CTSF*. In addition, there is an upstream region of ~29 kb in which both CEU and ASN cohorts show complete homozygosity (**Fig. 4b**), although this region is separated from R577X in the CEU cohort by several SNPs with evidence of recombination; this region contains the last 14 exons of the *BBS1* gene and the final exon of the *ZDHHC24* gene. To identify alternative candidates for the target of selection in these regions, we sequenced all coding exons and

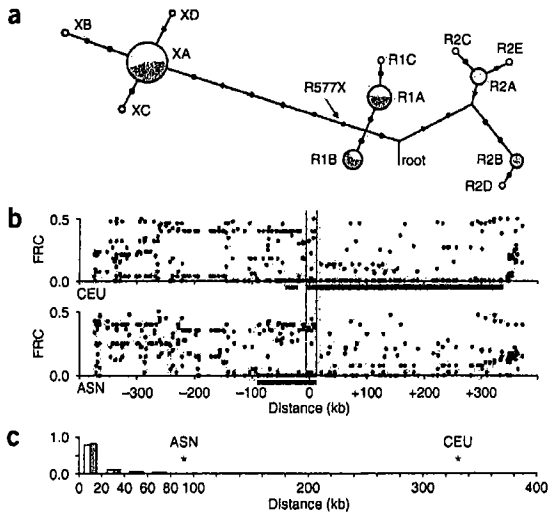

Figure 4 The 577X null allele is associated with low genetic diversity and high long-range LD in Europeans and Asians, suggestive of recent positive selection. (a) Haplotype genealogy in CEU and ASN samples. Grey and white circles denote haplotypes, with areas of white and gray proportional to frequency in CEU and ASN cohorts, respectively. Filled circles indicate intronic (black), synonymous (green) and nonsynonymous (purple) mutations. The likely position of the R577X substitution is shown, but note that the precise order of mutations along this branch cannot be inferred. The root of the tree is inferred from the chimpanzee sequence. (b) Pattern of long-range LD associated with R577X. The inferred fraction of recombinant chromosomes (FRC) for each HapMap SNP is plotted against physical distance for the 577R (blue) and 577X (red) alleles. Lengths of complete homozygosity (LCH) associated with the 577X allele are shown as red bars. An upstream region of homozygosity shared by ASN and CEU but noncontiguous with R577X is shown in orange. The region of overlap between the contiguous LCH in the two cohorts is shown (dotted lines). (c) Distribution of LCH values from a matched genome-wide set of >17,000 SNP alleles in the CEU (white) and ASN (gray) populations. LCH values for 577X in the CEU and ASN cohorts (asterisks) are clear outliers.

proximal splice sites in both areas, excluding exons of the *ACTN3* gene (as any variant in *ACTN3* linked to the sweep would be silenced by the 577X allele), in individuals homozygous for each of the three major haplotype clusters from both the CEU and ASN populations. We identified a total of 15 segregating variants by this analysis, none of which was both putatively functional and linked to the signal of selection (**Supplementary Table 4** online). In addition, we genotyped two previously reported nonsynonymous variants in the *CTSF* gene in 30 CEU and ASN individuals and found them to be monomorphic in these populations. Thus, although we cannot rule out variants in noncoding regions, the best candidate for the target of selection is the 577X allele, which has a known biological effect and which is linked to the signal of selection.

Using a recently described approach[9], we estimate that the sweep in CEU began ~15 kya, whereas in ASN the sweep is considerably older, dating to ~33 kya. The failure of the 577X allele to achieve fixation (that is, failure to reach a frequency of 100%) in either population may simply reflect inadequate time. However, even weak positive selection would be expected to increase the frequency of a beneficial allele to fixation within as little as a few hundred generations[8], substantially less than the estimated age of the 577X allele in the CEU and ASN populations. This may indicate the presence of more complex countervailing or balancing selective pressures acting on the R577X polymorphism and preventing a rapid sweep to fixation within these populations.

In conclusion, we have found that α-actinin-3 deficiency alters skeletal muscle metabolism and increases endurance performance in a knockout mouse model and that the *ACTN3* 577X null allele has been selectively favored in modern humans adapting to the Eurasian environment. We propose that the shift toward more efficient aerobic muscle metabolism associated with α-actinin-3 deficiency underlies the adaptive benefit of the 577X allele and, in turn, potentially explains the overrepresentation of the 577XX genotype among elite endurance athletes[3].

METHODS

Generation of knockout mouse. Figure 1a shows the strategy used to generate a null allele in the mouse *Actn3* gene. We amplified two regions flanking exons 2–7 of the *Actn3* gene using the Expand Long Template PCR system (Roche), with primers containing either *NotI* or *ClaI* restriction sites. These sites were used to clone the two flanking regions into the vector pPGKneoDTA on either side of the neomycin resistance cassette (*neo*).

The linearized vector was electroporated into R1-129 embryonic stem (ES) cells. Screening for the presence of successful homologous recombinants at the *Actn3* locus was performed by amplification with primers KO5′F and KOR5 and digestion with *EcoRI* (**Fig. 1a**). Recombinant ES clones were regrown, injected into cultured BALB/c blastocysts and transferred into pseudopregnant foster mice. Chimeric males identified by brown coat color were bred with 129 females. Heterozygous 129 mice were bred further to produce homozygous knockout mice. Mice were genotyped using by DNA isolated from tail snips, using the same protocol as for ES cells.

All animal experiments were performed in accordance with institutional and Australian National Health and Medical Research (NHMRC) guidelines.

Immunohistochemistry and immunoblotting. Antibodies to the α-actinins were a gift (see Acknowledgments). α-actinin-2 was analyzed using the antibody 4B3 at 1:1,000 for immunohistochemistry (IHC) and 1:200,000 for protein blot, and α-actinin-3 was analyzed using 5B3 at 1:1,000 for IHC and at 1:4,000 for protein blot. IHC for myosin heavy chain 2B was performed using the antibody 10F5, neat. Immunoblotting for mitochondrial porin used the antibody 20B12 (Molecular Probes) at 1:5,000, and immunoblotting for cytochrome c oxidase used 20E8 (Molecular Probes) at 1:2,000. Immunohistochemistry and immunoblotting were performed as described previously[10,11].

Histochemistry. NADH-tetrazolium reductase (NADH-TR) and succinate dehydrogenase (SDH) staining was carried out using standard protocols (**Supplementary Methods** online). Both NADH-TR and SDH stains were performed on sections of whole quadriceps muscle from ten $Actn3^{+/+}$ and ten $Actn3^{-/-}$ mice. In both cases, all $Actn3^{+/+}$ and $Actn3^{-/-}$ mice could be distinguished from one another by two independent observers unaware of their genotype, judging solely from staining intensity and distribution.

Quantitative image analysis of NADH-TR staining was performed on five $Actn3^{+/+}$ and five $Actn3^{-/-}$ male mice using Adobe Photoshop as described in **Supplementary Methods**. Statistical significance was assessed using the Mann-Whitney *U* test in SPSS.

Metabolic enzyme assays. Lactate dehydrogenase (EC 1.1.1.27) and citrate synthase (EC 4.1.3.7) activities were measured spectrophotometrically by modifications of previously described methods[12,13].

Intrinsic exercise capacity test. Mice were tested for intrinsic endurance capacity using a modified version of a previously described protocol[14]. Briefly, 7- to 9-week-old male mice were placed on a motorized treadmill (Columbus Instruments) set at a 15° incline with a baseline speed of 10 m/min. A negative stimulus (a stiff-bristled brush) was placed at the rear of the treadmill. Speed was increased by 1 m/min every 2 min until mice were exhausted, defined as the inability to extricate themselves from the negative stimulus three times within two minutes. Speed and time of exhaustion were recorded, and total distance calculated. All mice were tested by observers unaware of genotype.

Sequencing of R577X region. Ninety-six unrelated DNA samples from the International HapMap Resource were obtained from Coriell Cell Repositories: 32 from CEU (CEPH Utah residents with ancestry from northern and western Europe), 32 YRI (Yoruba from Ibadan, Nigeria), 16 CHB (Han Chinese from Beijing) and 16 JPT (Japanese from Tokyo). A list of the samples used is provided in **Supplementary Table 1**. In addition, the region was sequenced from one chimpanzee DNA sample.

A region of genomic DNA spanning ~3.8 kb (chromosome 11, positions 66082752–66086604 from NCBI build 36.1 of the human genome) centered on the R577X polymorphism (dbSNP rs1815739) was amplified in two overlapping fragments and was fully sequenced using 20 primers (**Supplementary Table 2**). Trace files were trimmed and aligned with a reference sequence using Sequencher (GeneCodes) and manually scanned for polymorphisms. Inference and analysis of haplotypes is described in **Supplementary Methods**.

Long-range LD analysis. Phase 2 genotype data from the CEU and ASN cohorts were downloaded from the HapMap website and were filtered to generate a set of all autosomal SNP alleles with frequencies within 10% of the frequency of 577X and local deCODE estimated recombination rates[15] within 0.05 cM/Mb of the estimated recombination rate of the R577X region. This matched data set contained 17,004 alleles for the CEU cohort and 17,051 alleles for ASN. Because the relative allele frequency range spans 0.5, both alleles were included for some SNPs.

A custom Python program (see URLs section below) generated using PyEvolve version 0.89.9 (ref. 16) was used to calculate the length of complete homozygosity (LCH) for each of the alleles in the matched set for both CEU and ASN, using the total HapMap data set, excluding monomorphic SNPs. The algorithm identified individuals who are homozygous for each target allele and determined the length of the largest region spanning the target SNP in which all these individuals are homozygous for the same allele at all other SNPs; this distance was defined as the LCH.

In the ASN cohort, the LCH for the 577X allele was interrupted by a single heterozygous genotype at rs597626 in sample NA18612; we re-genotyped this individual and found this to be a genotyping error in the HapMap database. An error report has been submitted to HapMap.

For **Figure 3b**, the fraction of recombinant chromosomes (FRC) was calculated using a previously described approach[8]. Briefly, the complete HapMap genotype data set for a 750-kb region centered on R577X was downloaded, and individuals were separated by R577X genotype. Heterozygotes were discarded, and individuals homozygous for the alleles encoding Arg577 ('577RR individuals') and individuals homozygous for the alleles encoding the stop codon ('577XX individuals') were analyzed separately. If

no recombination had occurred between R577X and a target SNP in the region, that SNP was monomorphic in individuals homozygous for one allele of R577X (that is, FRC = 0). If recombination had occurred, the minor allele frequency of the target SNP in individuals homozygous for one allele of R577X served as an estimate for the FRC.

Ages of the selective sweeps in the CEU and ASN cohorts were estimated using a recently described method[9] based on the extent of decay of LD associated with the 577X allele. These estimates should be regarded as tentative, as they rely on a number of assumptions of uncertain validity[9].

Analysis of *CTSF, BBS1* and *ZDHHC24* genes. To minimize the possibility that the true target of selection in this region was a functional variant outside of *ACTN3*, we sequenced all coding exons of the *CTSF, BBS1* and *ZDHHC24* genes contained within the regions of LCH overlapping between CEU and ASN, along with >50 bp of flanking intronic sequence for each exon, in six individuals selected to represent homozygotes for each of the three major haplogroups from both the CEU and ASN cohorts. Primers used are listed in **Supplementary Table 2**. We also genotyped the only two reported nonsynonymous polymorphisms in the *CTSF* gene (rs11550508 and rs28464796) in 13 RR individuals and 17 XX individuals from our sequenced CEU and ASN cohorts, using direct sequencing or RFLP assays (**Supplementary Methods**).

Accession numbers. dbSNP: rs1815739 (R577X polymorphism).

URLs. Code for the custom Python program is available at http://jcsmr.anu.edu.au/org/dmb/compgen/software.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

D.G.M., N.Y., J.W.H., F.A.L. and P.W.G. generated the knockout mouse; D.G.M., J.T.S., K.G.Q., J.M.R., N.Y., M.R.E., Y.B., A.J.K. and E.C.H. analyzed the knockout mouse phenotype; D.G.M., J.M.R., G.A.H. and S.E. performed the evolutionary analysis; D.G.M. and K.N.N. designed the studies and wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturegenetics/.

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The ACTN3 (R577X) genotype is associated with fiber type distribution

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Running head: ACTN3 (R577X) polymorphism and fiber type distribution

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Abstract

Alpha-actinin-3 is a Z-disc structural protein found only in type II muscle fibers. The X-allele of the R577X polymorphism in the ACTN3 gene results in a premature stop codon and α-actinin-3 deficiency in XX homozygotes. Former studies reported a strong association between the R577X polymorphism and elite athletes' muscle-power performance. About 45% of the fiber type proportions are determined by genetic factors. The ACTN3 variant could be one of the contributing genes in the heritability of fiber type distribution through its interaction with calcineurin. Aim of this study was to quantify the association between the polymorphism and muscle fiber type distribution and fast-velocity knee extension strength. Ninety healthy young men (18-29 yr) were genotyped for ACTN3 R577X. Knee extensor strength was measured isometrically (45°), and at different dynamic velocities (100-300°/s) on a programmable dynamometer. Twenty-two XX and twenty-two RR subjects underwent a biopsy of the right vastus lateralis muscle. Fiber type composition was determined by immunohistochemistry. Homozygotes for the R-allele show significantly higher relative dynamic quadriceps torques at 300°/s, compared to XX carriers ($p<0.05$). Fiber type characteristics differed significantly between the two genotype groups. The percentage surface and number of type IIx fibers was greater in the RR than the XX genotype group ($p<0.05$), and α-actinin-3 protein content is systematically higher in type IIx compared to type IIa fibers (staining intensity ratio IIx/IIa=1.17). This study shows that the mechanism, by which the ACTN3 polymorphism has its effect on muscle power, might rely on a control function of fiber type proportions.

Keywords: alpha-actinins, muscle genetics, immunohistochemistry, fiber typing, polymorphism

Introduction

The ACTN3 gene encodes the protein α-actinin-3. Alpha-actinin-3 is an actin-binding protein which is structurally related to dystrophin (6). In humans, two genes encode for skeletal muscle α-actinins: ACTN2, which is expressed in all skeletal muscle fibers, versus ACTN3, the expression of which is limited to fast-twitch muscle fibers (100% of type IIb/x fibers and 50% of type IIa fibers) (16, 17). Alpha-actinins are important structural components of the Z-membrane (1) where they form the crosslink between the thin actin filaments. They have a static function in maintaining ordered myofibrillar arrays and a regulatory function in coordinating myofiber contraction (13). Interestingly, in European Caucasian populations about 18% of the individuals are fully α-actinin-3 protein deficient due to homozygosity for a premature stopcodon polymorphism in the ACTN3 gene (Chrom 11. pos. 66084671, C→T, R577X, rs1815739). However, this deficiency does not result in a disease phenotype or muscular functional impairment (10, 11, 17). Still, a number of studies have provided data to indicate that there is a positive association between the presence of the R-allele and the capacity to perform high power muscle contractions. On the other hand, the X-allele might predispose for better endurance exercise performance (14, 15, 24). Accordingly, Yang et al. found in a sample of white elite athletes a higher frequency of the 577R allele in both male and female sprinters, whilst elite endurance athletes exhibited a slightly higher frequency of the XX genotype (24).

Alpha-actinins interact with themselves, structural proteins of the contractile machinery, metabolic enzymes and signalling proteins (reviewed in 10), amongst them also members of the Z-line localized calsarcin family (8). These bind to calcineurin - Ca^{2+}- and calmodulin-dependent protein phosphatase - which is a signalling protein and is hypothesized to play a role in the determination of muscle fiber type and muscle hypertrophy (10), although it does not seem to be implicated in muscle fiber growth in regenerating muscle (19). Semsarian et al. showed that in reaction to intracellular calcium mobilisation, calcineurin is activated. The

latter in turn causes a nuclear translocation of the transcription factor nFATc1. In rats, the activation of calcineurin mobilises satellite cells and causes a switch to a more glycolytic metabolism (20). On the contrary, Chin and co-workers reported that the activation of calcineurin selectively up-regulates slow-fiber-specific gene promoters (2).

Based on genetic epidemiological studies, about half of the variability in fiber type distribution in human muscles is determined by genetic factors (21). Through its interaction with calcineurin, polymorphisms in the ACTN3 gene could conceivably contribute to heritability of fiber type distribution. The force-generating capacity of type II muscle fibers at high velocity, the speed of movements and the capacity to adapt to training are all strongly genetically influenced (24). The contribution of genetic factors in strength measures in part varies according to the angle, contraction type and to some extent contraction velocity (23). Contractile property differences according to the presence/absence of α-actinin-3 in sarcomeres of fast-type muscle fibers might also contribute to individual differences in power output.

Currently not much is known about the effect of the α-actinin-2/3 protein content in the muscle. North et al. observed in 1996 that the α-actinin-2/3 protein content is fiber type dependent. They show that while α-actinin-2 is found in all skeletal fibers; α-actinin-3 is present in only a subset of type II fibers (all type IIb fibers and 50% of the type IIa fibers), although no numeric information was reported (16).
This issue raises the question whether the content of these proteins differs between individuals and if so, whether this difference can explain variation in performance.

The primary purpose of this study was to investigate the relationship between the ACTN3 (R577X) genotype and muscle fiber type distribution in humans on the one hand and the capacity of force generation of the muscle fibers (at different velocities) on the other hand.

The secondary aim was to investigate the relationship between fiber type specific α-actinin-2 and 3 protein levels and skeletal muscle performance.

Because of the exclusive prevalence of α-actinin-3 in fast glycolytic (type II) muscle fibers, and the interaction between the α-actinins and calcineurin (with its likely function in fiber type determination) we proposed the following hypotheses.

(1) Subjects with α-actinin-3 deficiency have lower baseline muscle power than subjects with α-actinin-3; this difference in dynamic strength becomes more obvious with increased velocity of contraction; (2) ACTN3 is essential for the differentiation and structural traits of muscle fibers and explains part of the inter-individual differences in fiber type distribution; (3) the observed differences in power output can at least in part be explained by a difference in α-actinin-2/3 protein content.

Materials and Methods

Subjects

Ninety healthy young males (age: 21.7± 2.3 years; body weight: 73.3 ± 8.6 kg) gave written consent to participate after being fully informed of the study protocol and procedures. The Ethics Committee of the Faculty of Medicine of K.U.Leuven approved the study protocol. All experiments were conducted in conformity with the principles of the declaration of Helsinki. The subjects were recruited by announcements amongst the local student population. Inclusion criteria on admission were: (1) male (2) age between 18 en 30 yrs (3) in good health. Exclusion criteria were: (1) acute or chronic disease (2) consistent intake of medication or nutrition supplements of any kind during a period of 6 months prior to the study (3) any medical condition that might contra-indicate high-intensity exercise (4) a prehistory of consistent resistance training in a period of 12 months prior to the study.

Study protocol

The study was performed in 2 phases. In phase I the relationship between the ACTN3 (R577X) polymorphism and muscle strength was studied in the total group (n=90) of subjects. In phase II, a subgroup was used to compare muscle fiber type distribution and muscle strength between RR (n=22) and XX (n=22) ACTN3 homozygotes. Furthermore, in the latter subgroup muscle α-actinin-2/3 protein content was also determined.

In phase I, subjects reported twice to the research center within a 3-week period. In the first session a blood sample was taken from an antecubital vein to be used for DNA extraction and SNP genotyping (RT-PCR, Applied Biosystems®). In the 2nd session the subjects underwent a series of anthropometric measurements. Thigh circumference (TC) and skinfold (TS) measured at half the distance from groin to patella was used to estimate m. quadriceps cross sectional area (CSA), using the following equation: CSA= (2.52*TC) - (1.25*TS) - 45.13 (9). Percentage body fat (%), fat mass (kg) and fat free mass (kg) was assessed by a Bio-

electric Impedance technique (Biodynamics, Model 310e, Bodycomposition Analyzer, Seattle, Washington).

After a short warm up session on a cycle ergometer, static and dynamic torques of the knee-extensor muscles were measured on a self-constructed computerized active isokinetic dynamometer (servomotor SEW Eurodrive CM90, Bruchsol, Germany). Subjects pushed against a lever arm and the exerted torque was directly measured using a calibrated torque transducer (type 1605, Lebow® Products Inc., Troy, USA, 0.05% accuracy level) mounted in the axis of the system, which was aligned to the axis of the knee joint. After positioning the subject in the backward inclined chair (30°), alignment of the knee and fixation of upper body and upper leg, the lower leg was fixed to the lever arm at the level of the ankle. First, subjects were allowed to get familiarized with the testing device applying submaximal efforts on one static,5 repetitions of dynamic extension movements at different speeds (100°/s and 400°/s) as well as one eccentric contraction (20 s rest between contractions). The start of each contraction was indicated by an audible and vibrating signal of the lever arm and after reaching the end position, the leg was passively returned to the starting position. After familiarization, the exerted torques during maximal voluntary contractions was measured. First, the average of two maximal isometric extensions (5 s) at 45° knee flexion was taken as static torque. Dynamic torques at 100°/s, 200°/s, 300°/s and 400°/s were defined as the registered torque (at 45°) during one extension movement (from 85° to 5° knee angle, with 0° as extended leg position) at each speed. Finally, eccentric torque was measured during a single maximal knee extension contraction while knee flexion was forced by the dynamometer at an angular velocity of 100°/s. About 15% of subjects were unable to perform qualitatively correct extensions at 400°/s (with very low torque values), we therefore chose to exclude this variable from the analyses.

Finally, total amount of physical activity was assessed by questionnaire (number of hours/week), furthermore, type of sports activities and recreational/competitive level was registered..

Phase I of the study yielded 22 XX carriers, who were ('ranked') pair-matched by 22 subjects with the RR genotype in order to obtain 2 homozygote groups (XX vs. RR) with identical distributions for maximal isometric muscle strength and fat free mass. In case several subjects were a candidate match for a subject homozygote for the X-allele, a random selection was made. Subjects reported to the laboratory in the morning after an overnight fast. A muscle biopsy was taken from the right vastus lateralis muscle under local anaesthetic (2-3 ml lidocaine®) using a Bergström type needle through a 5 mm incision in the skin. After being freed from any visible non-muscle material, part of the muscle sample was immediately frozen in liquid nitrogen, and the remaining part was mounted in embedding medium (Tissue-Tek, Sakura FineTek, Zoeterwoude, The Netherlands) cooled in isopentane. Samples were stored at -80℃ until analyzed for fiber type distribution and α-actinin-2/3 protein content at a later date.

Analyses of blood and muscle samples

SNP genotyping - DNA was extracted using the Chemagic DNA Blood Kit on an automated Chemagic Magnetic Separation Module I (Chemagen®, Baesweiler, Germany) and a Multiprobe I (PerkinElmer®, Waltham, Massachusetts, USA) robotic station.

Genotyping was performed using a TaqMan SNP genotyping assay (Applied Biosystems®), containing a 20x mix of unlabeled PCR forward and reverse primers as well as a VIC and FAM labelled allele discrimination probe. The Assay ID was C__590093_1. Real-time qPCR was carried out in a 20 μl reaction mixture with 5 μl cDNA, 4 μl RNase-free water, 1 μl of 20x TaqMan SNP genotyping assay mix and 10 μl of the 2x Taqman universal PCR master mix (Applied Biosystems®). Amplification and detection were performed using the ABI PRISM 7300 sequence detection system (Applied Biosystems®). Thermal cycling conditions were 10 min at 95℃ followed by 40 two-step cycles including 15s denaturation at 92℃ and 60s annealing/extension at 60℃. All reactions were set up manually and allele calling was done using SDS 1.3 software®.

Muscle histochemistry - Serial sections (4μm) from biopsy samples were collected on uncoated glass slides. Briefly, cryosections were fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS). Slides were rinsed for 2 x 5 min with wash buffer (0.5% BSA in PBS), treated with 10mM NH_4Cl and washed again (2 x 5 min). Slides were prehybridized in 1% BSA in PBS for 30 min. Sections were then incubated overnight at 4℃ with the primary antibodies. The incubation was followed by 3 x 5 min washes with wash buffer, after which the appropriate conjugated antibodies were added. Finally, the sections were washed again (3 x 5 min in wash buffer) and coverslips were mounted with fluorescent mounting medium (DakoCytomation, Carpinteria, CA, USA). For muscle fiber typing we used primary antibodies directed against human myosin heavy chain I and IIa (A4.840 and N2.261 supernatant from Developmental Studies Hybridoma Bank at the University of Iowa, IA, USA). The primary antibodies against α-actinin-2 and α-actinin-3 were sera and affinity purified rabbit polyclonal antibodies raised against amino-terminal peptides, rabbit anti-α-actinin-2 rod raised against a peptide from the central rod domain, and monoclonal mouse anti-merosin M-chain (16). Fiber type specific stainings for α -actinin-2 and α-actinin-3 protein content were performed in separate experiments. Pilot experiments revealed no cross-reactions between different primary and secondary antibodies.

The secondary antibodies for fiber typing were FITC anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL, USA) and Alexa Fluor 350 anti-mouse IgG1 (Molecular Probes, Leiden, the Netherlands) for type I and type IIa fibers, respectively, and anti-rabbit IgG (Acris GmbH, Germany) for α-actinin-2/3. Slides were examined using a Nikon E1000 fluorescence microscope (Nikon, Boerhavedorp, Germany) equipped with a digital camera. Epifluorescence signal was recorded using a FITC, DAPI and TxRed filter for type I, IIa muscle fibers and α-actinin-2/3 protein content, respectively, using standardized camera and microscope settings. Captured images (x 20 magnification) were processed and analyzed using Lucia G software (LIM, Prague, Czech Republic). Fibers, negatively stained for type I and type IIa were qualified as type IIx fibers. To eliminate inter-assay variation

samples aimed for mutual comparison were consistently included in the same assay. Background correction was performed by adding negative control samples in each assay.

Forty-three muscle samples were included in the fiber typing analysis. The number of fibers analyzed per sample was 170±11. The intra-assay coefficient of variation for fiber type proportions and surface areas was 5%.

Statistical analysis

The statistical analyses were done with SAS 9.1® and Statistica 6® software.

An ANOVA (Analysis of Variance) was performed to evaluate an association effect between the strength phenotypes and the three genotype groups; to investigate a possible co-dominant effect. An estimation of the cross-sectional area of the quadriceps was used as a covariate in an ANCOVA analysis. To determine possible dominant allele effects, independent sample t-tests were performed (have X allele *versus* have no X-allele; and have R-allele *versus* have no R-allele). To test for a possible contraction velocity interaction by different ACNT3 genotype groups, a repeated measures ANOVA was applied to raw and relative torque values at different contraction speeds.

T-tests were also used to determine differences between the two selected genotype groups (RR *versus* XX, n=43) in the fiber typing analyses. Pearson (or Spearman) correlations were used to study correlations between staining values for α-actinin-2/3 fiber-specific protein content and performance or fiber composition phenotypes. Multiple regression analysis was added to implement the cross sectional area of the quadriceps and fiber type proportions as covariate factors. A probability level (p) less than 0.05 was considered statistically significant. All data are expressed as means ± SE.

Results

Prevalence of ACTN3 R577X polymorphism and body composition- The proportion of XX, RX and RR genotypes in the study sample was 0.24, 0.44 and 0.31, respectively. These frequencies are in Hardy Weinberg equilibrium (Chi^2 value=0.95, p=0.33). The allele frequencies are 0.47 and 0.53, for the X-allele and R-allele respectively. Body composition characteristics as well as estimated quadriceps muscle cross-sectional area were similar between the three genotypes (Table 1).

Muscle fiber type distribution – Muscle fiber type distribution was measured in biopsy samples obtained from m. vastus lateralis (Table 2). On average the relative fraction of type I, IIa and type IIx fibers was 52%, 36% and 12%, respectively, in the total group. Genotype-specific differences were found for the percentage number of type IIx fibers, which was ~5% higher in the RR than in XX genotype group (p=0.04). Given similar average surface area per type IIx fiber, the relative muscle surface area covered by type IIx fibers was also slightly greater in the RR than in the XX genotype group (p=0.03). There were no significant differences between the genotype groups for either type I or type IIa fiber number or surface area.

Alpha-actinin-2/3 protein content – Muscle α-actinin-2/3 protein content was determined using immunohistochemical assays (see Figure 1). Staining intensity for α-actinin-3 protein in type II fibers on average was 80% higher in RR than in XX, which confirms the identification of the polymorphism (p<0.001, data not shown). Staining intensity was similar between type I fibers in RR and any fiber type in XX, which indicates the lack of expression of α-actinin-3 protein in type I fibers (p=0.72) (data not shown). The expression of α-actinin-3 protein content was compared between type IIa and type IIx fibers in RR subjects exhibiting a sufficient number (>10) of type IIx fibers in the muscle sections (n=11; Figure 2). Staining intensity for alpha-actinin-3 protein content largely varied amongst the different subjects. However, compared with type IIa fibers, α-actinin-3 average red staining in type IIx fibers on average was ~17% higher (p=0.04), and this difference was consistent in all but one

individual. Average red staining for alpha-actinin-2 protein content was similar between the two genotype groups (data not shown).

Muscle strength – Muscle strength was measured as torque production during maximal static and dynamic knee extensions on an isokinetic dynamometer. As shown in Table 3 absolute torque production was similar between the three genotype groups for either static, dynamic concentric and eccentric contractions. However, dynamic torque production expressed relative to maximal static torque was different between genotypes for the highest contraction speed. At 300?s the RR genotype group showed higher relative knee extension torques than the XX group (p=0.04) (total study group, n=90). As shown in Figure 3 for the XX and RR groups, following the strength-velocity relationship, relative torque production decreased as contraction velocity increased. However, this decrease was greater in the XX than in the RR group (genotype by velocity interaction: p=0.06). Similar results were found when estimated quadriceps cross-sectional area, BMI or weight was included as a covariate. Within the RR genotype, there was no correlation between muscle α–actinin-3 staining intensity and torque output during high velocity (300?sec) muscle contractions, even after correction for differences in fiber type distribution in multiple regression analysis.

Discussion

The ACTN3 (R577X) polymorphism causes a complete loss of the α-actinin-3 protein in XX homozygotes. About 18 percent of European-ancestry populations are ACTN3 deficient, with no obvious related pathology. The polymorphism has been associated with elite athletic performance; the R allele was more common in sprint and power athletes, while the X allele was more frequent in endurance athletes (10, 11, 14, 15, 24). Through an interaction with the signalling protein calcineurin, Yang et al. proposed that α-actinin-3 might promote the formation of fast twitch fibers (24).

Here, we tested whether the R577X genotype is associated with baseline muscle strength (dynamic torque), though more specifically the association of this polymorphism with fiber type proportions and characteristics in healthy young men. We also documented fiber-type specific α-actinin-2/3 protein contents. We took blood samples from 90 men to determine their R577X genotype. These subjects performed several strength measurements at different velocities on a computerised isokinetic dynamometer. From 44 of these subjects (22XX and 22RR carriers) a muscle biopsy was taken for the analysis of fiber type proportions and fiber-type specific α -actinin-2/3 protein content using immunohistochemical assays.

The relative allele frequency of the 577X allele for our study population was 0.47. This is similar to the frequencies previously reported (3, 13, 14, 24). Our population frequency for XX homozygotes was 24%, which is slightly higher than the 18% found for Europeans by Yang et al. (24). This may be due to the specific characteristics of our research population, which consisted mainly of physically active young men (18-29 yrs).

We found no association between the ACTN3 genotype and anthropometric or body composition characteristics. These findings are similar to the study of Moran et al. (14).

XX homozygotes showed significantly less (relative) dynamic muscle power than homozygotes for the wild type allele (total group and subgroup analysis). In the total group analysis, heterozygotes were intermediate between both homozygotes for most of the quadriceps torque values, indicating a co-dominant gene action. These findings expand on earlier studies that mostly used a case-control approach (15, 25) including elite athletes. Our findings show an additive effect of each R allele to enhance power in healthy non-athlete young men (18-29 yrs).

Other research groups were not able to detect a significant increased muscular strength/power in the untrained state (3, 4), however, location (elbow joint), sample ethnicity and ages were different from the present study.

The major aim of our study was to investigate the role of the ACTN3 (R577X) polymorphism in the determination of fiber type characteristics. We hypothesized that the associations between the ACTN3 (R577X) polymorphism and fast-velocity isokinetic torque could be explained by increased type II muscle fiber differentiation (in RR carriers), and therefore the force delivering capacity of the type II muscle fibers in dynamic (ballistic) movements.

This role of ACTN3 could be through a binding of α-actinin-3 with calsarcins that interact with the signalling protein calcineurin, to promote the formation of fast twitch fibers (24).

Calcineurin, a serine-threonine phosphatase activated by Ca^{2+}-calmodulin, participates in signalling pathways important for gene regulation and biological responses to external stimuli in many organisms and in many types of cells (5, 18). Chin et al. (1998) showed that a signalling pathway that involves calcineurin controls fiber type specific gene expression in skeletal muscles. They identified a molecular mechanism by which different patterns of motor nerve activity selectively promote changes in gene expression to establish the specialized characteristics of slow and fast myofibers (2). Semsarian et al. (1999) concluded that calcineurin promotes a switch to a more glycolytic metabolism. In IGF-1 injected rats,

activated calcineurin mobilized satellite cells, increased activity of glycolytical enzymes and the end product of the glycolytical metabolism (lactate) (20).

However, contrary findings are described in the study of Swoap et al. (2000), where they show that active calcineurin is not sufficient to differentially regulate fiber type specific gene expression in whole muscle or in cell culture (22). Furthermore, Serrano et al. (2001) state that calcineurin activity in muscle fibers is required for the induction and the maintenance of the slow muscle gene program and the repression of the fast MyHC-IIx genes (19). Contrary to Serrano's findings, Michel et al. (2004) conclude that calcineurin signalling primarily leads to an adaptation towards a more metabolically efficient phenotype in response to increased muscle usage. Hence, calcineurin is more than a signalling agent for exclusive maintenance of slow type I fiber profiles in response to 'slow/chronic' patterns of nerve activation (12). Dunn et al. (2001) also present a more differentiated role for calcineurin than the sole maintenance of the slow gene program. They state that calcineurin signalling increases in all fiber types with increased nerve-mediated activity. Highly relevant to the findings in this study, most reactivity was found in the less active (IIx) fibers. The authors hypothesize that the transcription of proteins is only increased when activation is above the 'native levels' threshold. Cells that already have a high calcineurin activity (type I fibers) become less sensitive for relatively small amounts of increased nerve activity (7).

In this article, we show a possible role of the ACTN3 gene in the determination of fiber type distribution. We found a positive association between the ACTN3 RR genotype and the amount and fiber surface of fast, glycolytic fibers (IIx) in terms of percentage ($p<0.05$). These data are in agreement with Yang's hypothesis (24) that α-actinin-3 promotes the formation of fast-twitch fibers. These findings suggest that the mechanism, by which the ACTN3 (R577X) polymorphism has its effect on muscle power, might rely, at least in part, on the regulation of fiber type proportions. However, these findings do not exclude that other signalling pathways and interactions with metabolic enzymes also play a role in the α-actinin-3 specific effects on the regulation of muscle fiber type distribution in humans (10).

Finally, we hypothesized that the differences found in dynamic muscle torque might in part rely on a different amount of α-actinin-3 protein in the fast muscle fibers in the homozygotes for the R-allele. We were unable to confirm this hypothesis. We found no correlation between the amount of α-actinin-3 staining in the fast fibers and the muscle performance at high velocity. However, for the speed at 100°/sec and 200°/sec a trend towards a positive correlation was observed for both α-actinin-3 red staining in type IIa and type IIx fibers. Variability within the RR-group for high-velocity contractions is probably related to many more genetic and non-genetic factors, for which α-actinin-3 protein variability would be only one contributing factor. Probably the relatively small sample size of subjects in the RR-group with α-actinin-3 protein level information and limited effect-size of the α-actinin-3 protein level does not warrant definite conclusions.

North et al. (1996) included unpublished observations concerning the partition of α-actinin-3 amongst type II fibers (16). They observed that the protein is present in all the type IIb fibers and 50% of the type IIa fibers. In our study population of healthy young men, α-actinin-3 was present in all type IIa fibers of RR carriers, and we found systematic higher levels of α-actinin-3 in IIx fibers (staining intensity ratio of IIx/IIa: 1.17 (± 0.11)). We are not aware of any other study reporting such data. For reasons of improved reliability, the analysis was restricted to RR individuals with at least 10 IIx fibers, and these subjects seemed to be less physically active compared to those individuals with less than 10 IIx fibers. Generalization of these results might only apply to moderately active males, however, also in subjects with less than 10 IIx fibers, the same trend in increased α-actinin-3 staining in IIx was observed. Furthermore, in the overall group (n=90), hours of physical activity per week did not differ between genotype groups, and including hours of PA as a covariate did not fundamentally change the results of reported associations between absolute and relative dynamic torques.

Concerning the fiber-type specific amount of α-actinin-2 protein, we found no differences in staining between the two genotype groups. Therefore, in XX-homozygotes no extra compensation seems to occur concerning the lack of α-actinin-3. Literature shows that α-

actinin-2 and α-actinin-3 are structurally similar which makes it likely that α-actinin-2 is able to compensate for the lack of α-actinin-3 (13), as confirmed in the present study, however with lower capacities of high velocity contractility.

To conclude, in the present study we expand the existing hypothesis that the R allele enhances high velocity muscle tasks, to healthy young men (18-29 yrs). This study is the first to our knowledge to show an association between the ACTN3 (R577X) genotype and the fiber surface and number of fast glycolytic fibers (IIx), in terms of percentage in favour of the RR carriers. Fiber-type specific α-actinin-3 content measures indicate a 17% higher staining intensity, indicative for a true difference in protein expression in type IIx fibers compared to type IIa fibers.

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Table 1: Anthropometric characteristics by ACTN3 R577X genotype in total sample (n=90)

	577XX (n=22)	**577RX** (n=40)	**577RR** (n=28)
Height (cm)	181 ± 1.0	181 ± 0.9	179 ± 1.1
Body weight (kg)	74 ± 1.7	74 ± 1.6	72 ± 1.2
Body fat (%)	12.9 ± 0.7	13.3 ± 0.6	12.8 ± 0.7
Fat mass (kg)	9.6 ± 0.6	11.6 ± 1.9	9.2 ± 0.6
Fat free mass (kg)	63.9 ± 1.2	62.6 ± 1.5	62.0 ± 1.0
Estimated M. Quadriceps Cross-Sectional Area (cm^2)	69.1 ± 2.1	70.4 ± 1.5	69.0 ± 1.8

Mean ± SE are given. Body composition data are from a subgroup of the total sample (XX: n=19; RX: n= 40; RR: n= 26).

Table 2: Effect of ACTN3 R577X polymorphism on muscle fiber type composition

	577XX GENOTYPE (n=21)	577RR GENOTYPE (n=22)
Proportion of fiber types (%)		
Type I	55 ± 3	50 ± 2
Type IIa	35 ± 2	37 ± 2
Type IIx	9 ± 1	14 ± 2 *
Average surface area per fiber type (μm^2)		
Type I	4265 ± 165	4404 ± 204
Type IIa	5318 ± 243	5611 ± 330
Type IIx	4581 ± 272	5095 ± 349
Relative surface area per fiber type (%)		
Type I	51 ± 3	45 ± 2
Type IIa	40 ± 3	42 ± 2
Type IIx	7± 1	12 ± 2 *

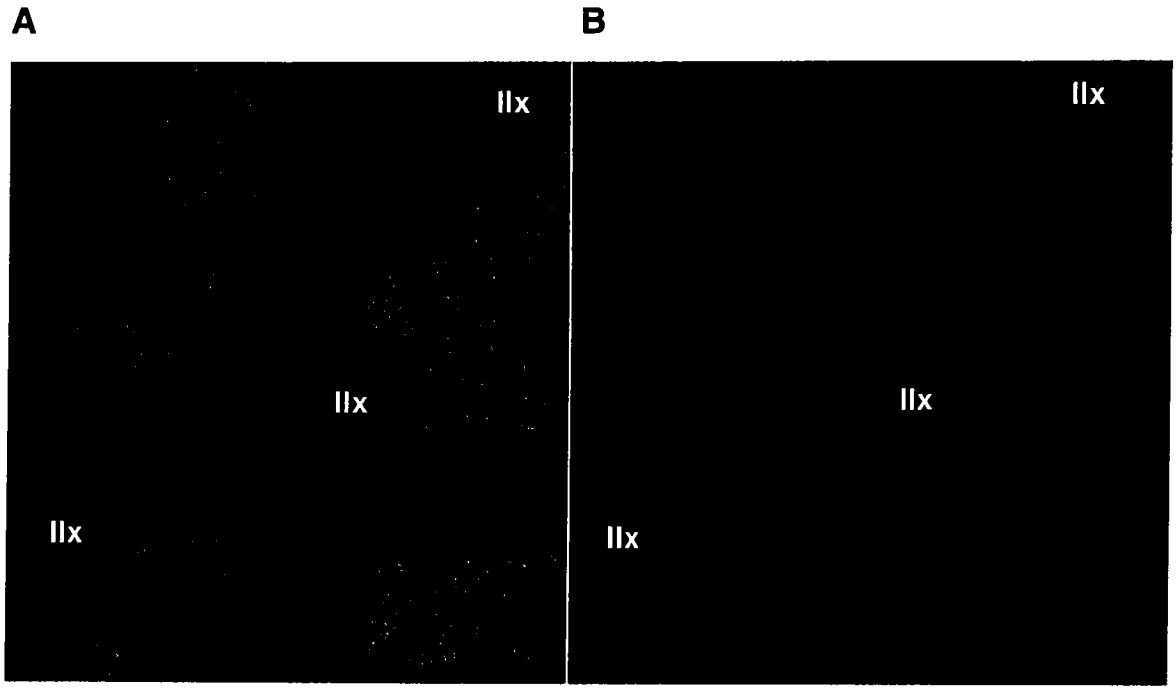
Values are means ± SE of 43 observations. Muscle fiber typing was performed by immunohistochemistry on biopsy samples obtained from m. vastus lateralis. See METHODS for further details.
* $p<0.05$ versus 577XX genotype.

Table 3: Effect of ACTN3 polymorphism on muscle strength

	577XX (n=22)	**577RX** (n=40)	**577RR** (n=28)
Absolute torque			
Static torque (45°)	211 ± 6	207 ± 4	195 ± 5
Dynamic torque			
100°/s	131 ± 6	137 ± 5	127± 6
200°/s	84 ± 6	89 ± 4	87 ± 5
300°/s	56 ± 6	59 ± 4	66± 5
Eccentric torque	219 ± 7	215 ± 3	212 ± 4
Relative torque			
100°/s	0.62 ± 0.02	0.66 ± 0.02	0.65 ± 0.02
200°/s	0.40 ± 0.02	0.43 ± 0.01	0.45 ± 0.02
300°/s	0.26 ± 0.03	0.28 ± 0.02	0.34 ± 0.02 *

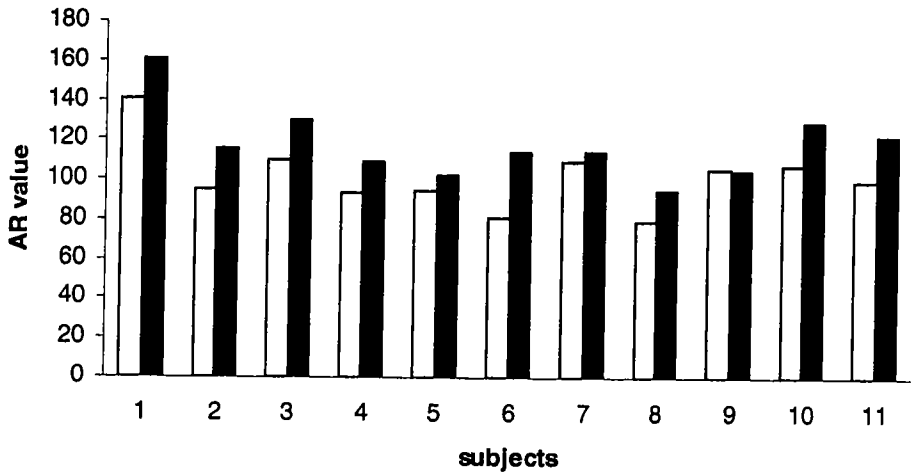
Data are expressed as mean ± S.E.M. of 90 observations. Absolute torques are expressed in Nm. See METHODS for further details. * p=0.04 versus 577XX genotype.

Figure 1: Immunohistochemical determination of fiber-type specific α-actinin-3 protein content



This figure shows the fiber type specific staining of α-actinin-3 in an RR-carrier subject. The bright blue fibers (panel A) correspond to type IIa fibers, which also contain the α-actinin-3 (bright red staining in panel B). There is no α -actinin-3 in the fibers staining green (type I fibers). The darker blue fibers (type IIx fibers) show relatively more α-actinin-3 protein content than the type IIa fibers in this subject.

Figure 2: Effect of fiber type on alfa-actinin-3 protein content



Data are individual values expressed in arbitrary units corresponding to red staining intensity corrected for background staining. Open bars, type IIa fibers; filled bars, type IIx fibers. See METHODS for further details.